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Histidine Decarboxylase and Histamine Binding in Rabbit Platelets.* (22573)

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The rabbit is the only species known which has a high concentration of histamine in the platelets(1). The origin of this histamine has hitherto been unknown. In this paper we show that rabbit platelets, *in vitro*, have a pronounced ability to decarboxylate C^{14} L-histidine and to bind the resulting C^{14} histamine in stable condition. The enzyme histidine decarboxylase has been prepared from rabbit platelets in soluble form and a study of its properties reported.

Methods. In some of the first experiments the entire buffy coat of rabbit blood was used; later, platelet preparations, practically free of blood cells, were obtained by the method of McIntyre.† These two preparations will be referred to as "buffy coat," and "isolated platelets," respectively. Blood collection and platelet isolation were done in silicone-coated glassware. Platelet preparations were incubated with 10 γ C^{14} L-histidine(2) activity 2.4 x 10^7 cpm per mg base. Incubations were carried out under nitrogen, at 37°C in 0.5 to 1.0 ml isotonic phosphate buffer, pH 7.4, containing 0.2% glucose. The C^{14} histamine formed was determined by isotope dilution assay using a rigorous extraction procedure which eliminates contamination by the excess C^{14} L-histidine(3). The histamine was counted as the dipicrate at infinite thickness in flow counters‡ and corrected for background only (background 20-22 cpm). Under the experimental conditions described above there is no significant destruction of C^{14} histamine by rabbit platelet preparations.

Demonstration of binding of new C^{14} histamine by rabbit platelets. Isolated platelets from 50 ml blood were divided into 3 portions and incubated with C^{14} L-histidine as described. At the end of the incubation period the tubes were centrifuged and platelets and supernates analyzed separately for C^{14} histamine. Platelets gave values of 126, 127 and

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† This unpublished method was kindly provided by Dr. Floyd McIntyre of the Abbott Laboratories. Rabbit blood is added to an equal volume of cold 2.5% sodium citrate solution and centrifuged in the cold for 5 minutes at 1200-1500 rpm. The cells are removed leaving a suspension of platelets.

‡ In one experiment (Fig. 2, upper curve) data were obtained by counting the samples in a Packard Tri-Carb Liquid Scintillation Spectrometer. For use in this counter it was necessary to prepare the dibenzenesulfonyl derivative of histamine(8) instead of the picrate.

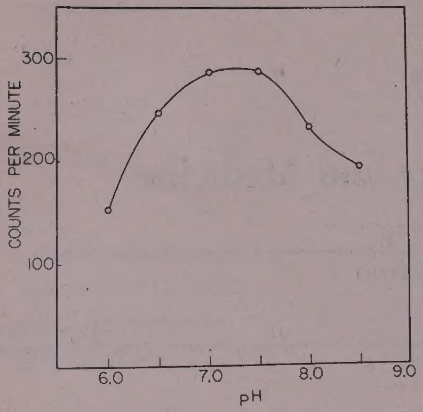

FIG. 1. Effect of pH on binding of histamine by rabbit platelets.

133 cpm averaging 129 cpm. Supernates gave values of 30, 32 and 33 cpm. Thus 80% of the newly formed histamine remains bound in the platelets.§ The remaining 20% escapes into the supernate either by diffusion, or by damage to the platelets, during incubation and centrifugation. If all the C^{14} L-histidine present were decarboxylated, the activity of the histamine dipicrate would be 10,500 cpm.

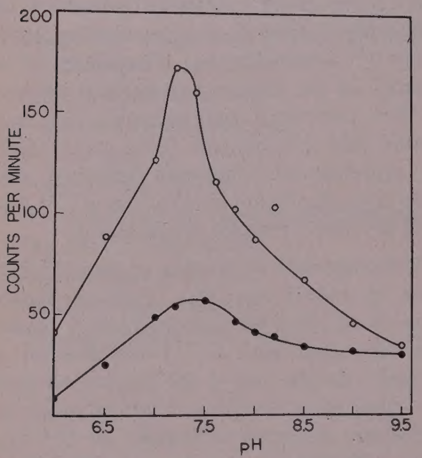

FIG. 2. Effect of pH on histidine decarboxylase activity. Upper curve, enzyme from isolated rabbit platelets; counts made with liquid scintillation counter. Lower curve, enzyme from rabbit buffy coat; counts made with flow counter.

Thus the values given above for the platelets correspond to about a 1.3% conversion of histidine to bound histamine.

Effect of pH on binding of C^{14} histamine. Aliquots of a suspension of rabbit buffy coat in saline were incubated with C^{14} L-histidine in phosphate buffers of various pH value. The effect of pH is shown in Fig. 1.

Effect of pH on histidine decarboxylase activity. Platelet preparations were suspended in saline, frozen and thawed 6 times, then centrifuged for 30 minutes at 25,000 x g. Aliquots of the supernate, free of particles, were added to buffers of various pH and incubated with C^{14} L-histidine as usual. Results are shown in Fig. 2.

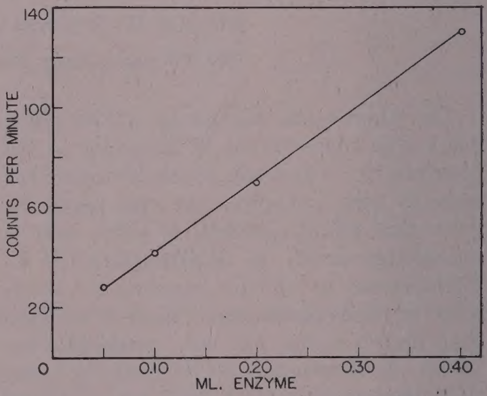

FIG. 3. Effect of concentration of histidine decarboxylase from isolated rabbit platelets on histamine formation.

Effect of concentration of histidine decarboxylase on rate of C^{14} histamine formation. Histidine decarboxylase from isolated platelets, prepared as described above, was used in varying concentrations for the incubation. Results are shown in Fig. 3.

Stability of histidine decarboxylase of rabbit platelets. Histidine decarboxylase was prepared from isolated platelets. The enzyme preparation was divided into 3 portions; one was assayed fresh while the other 2 were frozen, lyophilized and stored at —15°C. The latter were reconstituted by dissolving in distilled water and assayed at varying intervals of time. Triplicate assays of the fresh enzyme were 61, 64, and 68 cpm

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§ The buffy coats of human, rat and guinea pig blood were also tested for histidine decarboxylase activity; it was extremely low or absent in all cases.

TABLE I. Effect of Various Compounds on Activity of Histidine Decarboxylase of Isolated Rabbit Platelets.

Compound	Concentration, molar	Histidine decarboxylase activity*	Avg	% inhibition
Control		95, 98, 98, 100, 100, 104, 105	100	
Hydroxylamine	1×10^{-5}	105, 109	107	0
"	1×10^{-4}	43, 48	46	54
β-2-thienylalanine	$.6 \times 10^{-3}$	89, 89	89	11
Potassium cyanide	$.6 \times 10^{-3}$	50, 65	58	42
Semicarbazide	$.6 \times 10^{-3}$	23, 23	23	77

* Data were obtained from 2 experiments. In order to make a comparison, avg counts per min. of controls were multiplied by a factor to convert to 100. Other values in this table were obtained by multiplying the observed count by the same factor.

averaging 64 cpm. A sample stored for 2 days assayed 71, 73 and 73 cpm averaging 72 cpm. A sample stored 3 weeks assayed 52, 56 and 58 cpm averaging 55 cpm. Thus the enzyme can be lyophilized and stored for a few days without loss of activity. There appears to be a slight loss of activity in samples several weeks old.

Effect of inhibitors on the activity of rabbit platelet histidine decarboxylase. Inhibition of histidine decarboxylase from various mammalian tissues, principally rabbit kidney, has been extensively studied(4). The effect of several of these inhibitors on the activity of rabbit platelet histidine decarboxylase is shown in Table I.

Discussion. We have demonstrated that isolated rabbit platelets have the ability to decarboxylate C^{14} L-histidine and to bind the resulting C^{14} histamine in stable form. Conversion of histidine to histamine by particle-free extracts of isolated rabbit platelets is shown to be enzymatic by measurements of the effects on activity of pH (Fig. 2), concentration (Fig. 3) and inhibitors (Table I). In studies on histidine decarboxylase from other laboratories the sources of enzyme were complex tissues like kidney and liver(5) in which an undetermined amount of the enzyme was from cells possessing a histamine-binding mechanism. It is believed that the present paper is the first to describe the properties of histidine decarboxylase from a relatively homogeneous source which possesses not only the ability to synthesize histamine, but also the ability to bind it in stable form. The inhibitors of histidine decarboxylase reported by Werle(4) are also effective on the enzyme from rabbit platelets. There are quantitative differences, however. Rabbit platelet histidine decarboxylase has a pH maximum at 7.2 to 7.4. This is markedly different from the higher values, about pH 9.0, reported by earlier workers for histidine decarboxylase from rabbit kidney(6). In a recent paper by Graham, *et al.*(7) a large number of tissues were tested for histidine decarboxylase activity at pH 9.2. On the basis of our finding of maximum activity at pH 7.2 to 7.4, and low activity at 9.2 it would seem necessary to find the pH maximum for each tissue before comparison of histidine decarboxylase activity is made.

Summary. Rabbit platelets decarboxylate C^{14} L-histidine and bind the resulting C^{14} histamine in stable condition. Soluble histidine decarboxylase has been prepared from rabbit platelets and a study of its properties reported.

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Stimulatory Effect of Estrogen Pretreatment on 3β-Hydroxysteroid Dehydrogenase Activity of Rat Testicular Tissue. (22574)

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Mammalian endocrine organs which produce steroidal hormones have been observed (1) to possess the enzyme 3-β-ol-dehydrogenase, which catalyzes the conversion of Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids. This biosynthetic transformation is thought to be requisite for production of androgenic, gestogenic and adrenocortical hormones which possess in common the α, β-unsaturated ketone system in ring A of the steroid nucleus. Studies correlating the levels of 3β-ol-dehydrogenase activity of certain endocrine tissues with experimentally induced hormonal fluctuations would be expected to contribute information pertinent to a better understanding of biosynthetic mechanisms involving steroid hormone production.

The ability of testicular tissue from normal and estrogen pretreated rats to convert Δ^5-androstene-3β, 17β-diol to Δ^4-3-ketosteroid is here compared. Estrogen pretreatment was employed to effect what might be called a "chemical castration" induced via an inhibition of the pituitary output of gonadotrophins (2). The failure in testicular androgen production brought about by the estrogen pretreatment was evidenced by atrophy of the sex accessory organs. Consequently, a correlation may be made of the levels of 3β-ol-dehydrogenase activity of testicular tissue with the amount of androgenic hormone produced by that tissue.

Methods and materials. Estrogen Pretreatment. Male Sprague-Dawley rats ranging in weight from 160 to 195 g were divided into groups of 4 to 8 animals and fed commercial balanced diet and water *ad libitum*. All estrogenic substances were administered subcutaneously for 2 weeks. Diethylstilbestrol dipalmitate (DESDP) was given both as pellet implants and as a solution in cottonseed oil. The pellets were implanted under the skin in the lumbar region, one 5 mg pellet on each side of the animal. Estradiol-17β was injected as a solution in cottonseed oil. The dose of estrogen was administered in 0.2 ml of oil with injections being given daily except Sundays. On the 15th day of the experiment, the rats were sacrificed under chloroform. The testes, seminal vesicles and ventral prostates were excised, cleaned of adherent fat and weighed. The testes after weighing were immediately quick-frozen over dry ice and stored in a deep freeze chest until the 3β-ol-dehydrogenase activity could be determined. *Preparation of homogenates.* The connective tissue tunic of the testes was stripped off while the organ was in the frozen state. The testes from 2 or 3 rats were pooled, weighed and homogenized in the serum-buffer medium described below. After thorough homogenization, sufficient medium was added to adjust tissue concentration so that the desired amount of tissue to be incubated was contained in 1 or 2 ml of homogenate. *The 3β-ol-dehydrogenase activity* of testicular homogenates was ascertained on the basis of amount of α, β-unsaturated ketone formed during incubation with Δ^5-androstene-3β, 17β-diol as substrate. The α, β-unsaturated ketone was determined spectrophotometrically* by the intensity of absorption at 240 mμ. Testosterone was employed as a reference standard. One or 2 ml of the homogenate were added to flasks containing 25 ml of equal parts of bovine serum and phosphate buffer pH 7.4 to which was added 2 μM Δ^5-androstene-3β, 17β-diol, 6 μM DPN and 1 mM niacinamide. The flask contents were oxygenated for one minute, stoppered, and incubated in a water bath for 2 hours at 38°C on a revolving spoked drum. At the end of incubation period, the contents of the flask

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* The assistance of Madelyn Becker in spectrophotometric determinations is gratefully acknowledged.

TABLE I. Effect of Estrogens on Reproductive Organs of the Male Rat.

			Body wt (g), mean and range		Organ wt (mean ± S.E.)		
Treatment	Dose, μg/kg/day	No. of animals	Initial	Final	Testes	Seminal vesicles	Ventral prostate
None		6	178	235	2660 ± 128	191 ± 15	158 ± 17
			(160–195)	(218–266)	(1150 ± 84)†	(82 ± 7)	(68 ± 7)
DESDP	Pellet implant‡	7	177	181	1460 ± 322*	50 ± 8*	35 ± 9*
			(160–188)	(167–198)	(810 ± 176)	(30 ± 4)*	(19 ± 2)*
None		8	178	218	2770 ± 106	179 ± 8	176 ± 12
			(154–190)	(191–235)	(1270 ± 68)	(85 ± 6)	(80 ± 5)
DESDP	114	5	176	203	2580 ± 92	78 ± 12*	82 ± 19*
			(160–184)	(192–213)	(1280 ± 51)	(39 ± 6)*	(41 ± 9)*
None		6	170	223	2710 ± 95	240 ± 16	217 ± 18
			(162–182)	(204–248)	(1230 ± 54)	(113 ± 6)	(90 ± 6)
Estradiol-17β	.5	5	176	215	2520 ± 101	212 ± 18	173 ± 18
			(171–185)	(200–231)	(1190 ± 70)	(99 ± 8)	(82 ± 9)
	2	6	175	203	2690 ± 112	153 ± 13*	129 ± 7*
			(170–180)	(198–208)	(1330 ± 53)	(76 ± 5)*	(64 ± 4)*
	8	6	174	202	2680 ± 133	124 ± 12*	121 ± 8*
			(170–180)	(190–214)	(1330 ± 56)	(61 ± 5)*	(61 ± 4)*
	32	6	177	210	2470 ± 117	106 ± 22*	107 ± 13*
			(170–188)	(200–222)	(1180 ± 52)	(58 ± 6)*	(51 ± 9)*

* Statistically significant at the 5% level or less from the respective control mean.

† mg/100 g body wt and S.E.

‡ Two, 5 mg pellets implanted subcut. in lumbar region.

were boiled to stop enzymatic action. Procedure for extraction and chromatographic separation of the α, β-unsaturated ketone was as described by Wiswell and Samuels(3) with modifications as suggested by Samuels (personal communication). After boiling, the flask contents were extracted 6 times with 15 ml portions of ether. The pooled ether extracts were evaporated to dryness under nitrogen and the dried extracts were taken up in 30 ml of hexane and chromatographed on aluminum oxide. The fractions were eluted according to the following scheme: Fraction I, 30 ml hexane; Fraction II, 10 ml 10% chloroform in hexane; Fraction III, 75 ml 25% chloroform in hexane; Fraction IV, 50 ml chloroform. The α, β-unsaturated ketone content of Fraction III after preliminary purification was determined spectrophotometrically in 95% ethanol.

Results. Effects of diethylstilbestrol dipalmitate. Table I gives data on the effects of treatment on growth of animals and their reproductive organs. Alterations in testicular 3β-ol-dehydrogenase levels for corresponding groups are given in Table II. The subcutaneous implantation of two 5 mg pellets of DESDP to young adult male rats brought about a marked suppression of growth of the animals. Whereas control rats gained an average of 57 g/rat during the test period, DESDP treated males gained only 4 g/rat. The suppression of growth was accompanied by atrophy of testes and sex accessory organs. It should be pointed out, however, that the degree of testicular regression was only slightly greater than concurrently retarded growth rate of the animals.

The 3β-ol-dehydrogenase activity was elevated in the testicular homogenates from the DESDP pellet treated animals. The increase/g of tissue was of the order of 1.7-fold. However, since testes from treated rats were considerably smaller than control gonads, the amount of ketone formed by an amount of homogenate equivalent to the total paired testicular weight was no greater than that calculated for the control. It seemed imperative to determine to what extent DESDP would affect the 3β-ol-dehydrogenase activity of testicular homogenates when dose levels of this estrogen were given which would not alter the gross morphology of the testes yet be sufficiently large to curtail androgen production

TABLE II. Effect of Estrogen Pretreatment on 3β-ol-Dehydrogenase Activity of Rat Testicular Homogenate.

			α,β-unsaturated ketone formed (testosterone equivalents)			
Treatment	Dose, μg/kg/day	No. of determinations	μg/g tissue/2 hr	Ratio, control: test	μg/pair testes/2 hr	Ratio, control: test
None		2	459		1068	
DESDP*	Pellet†	2	776	1:1.7	1130	1:1.06
None		4	392		1087	
DESDP	114	4	662	1:1.7	1709	1:1.6
None		4	348		943	
Estradiol-17β	.5	2	363	1:1.04	915	1:0.97
	2.	2	754	1:2.2	2012	1:2.12
	8.	2	668	1:1.9	1790	1:1.9
	32.	2	1250	1:3.6	3090	1:3.3

* Diethylstilbestrol dipalmitate.

† Two, 5 mg pellets implanted subcut. in lumbar region.

as manifested by the degree of sex accessory organ atrophy. It had been previously observed(4) that a daily dose of DESDP equivalent to 114 μg/kg (20 μg/175 g rat) brought about a statistically significant regression in seminal vesicle and ventral prostatic growth at the end of 2 weeks but did not measurably alter the mass of the testes. This dose was selected for the present study. The expected organ weight changes with the use of 114 μg/kg/day of DESDP were obtained (Table I). The degree of atrophy of the sex accessory organs in this case was not maximal as judged by a comparison with the organ weights in the pellet implanted series; nevertheless, it was statistically significant ($P < .05$). The conversion of Δ^5-androstene-3β, 17β-diol by the testicular homogenates was again demonstrated to be increased by the DESDP pretreatment. The extent of stimulation in terms of μg of α, β-unsaturated ketone formed per gram of tissue per 2 hours was, for all practical purposes, identical with that seen with the DESDP pellet implants. Since there was no change in total testicular weight due to the DESDP pretreatment, this change in 3β-ol-dehydrogenase activity is accepted as stimulation of the enzymatic mechanism.

Effect of estradiol-17β pretreatment. Experiments were performed in which natural estrogen, estradiol-17β, was administered subcutaneously in doses of 0.5, 2, 8 and 32 μg/kg/day, 6 days a week for 2 weeks. The growth of rats on these dose levels appeared to be slightly suppressed. No significant atrophy of testes was observed, but a regression in weight of sex accessory organs occurred which in degree was proportional to the dose. A remarkable stimulatory effect on the 3β-ol-dehydrogenase activity of testicular homogenates was effected by estradiol, particularly when given at 32 μg/kg. This amount was observed to bring about a 3- to 4-fold increase in the ability of testicular tissue to form α, β-unsaturated ketone when incubated with the substrate, Δ^5-androstene-3β, 17β-diol. At 0.5 μg/kg daily, estradiol-17β did not affect the enzymatic activity of the testes, nor was it capable of evoking a statistically significant curtailment in production of androgenic hormone by the testes. Estradiol-17β, at 2 and 8 μg/kg, exerted an intermediate effect which in terms of increased α, β-unsaturated ketone production was slightly greater than that found for DESDP pretreated rats.

Discussion. The enzyme 3β-ol-steroid-dehydrogenase, obtained from mammalian endocrine tissues, is assumed to participate in the biological synthesis of the steroid hormones which possess the α, β-unsaturated ketone structure in ring A of the steroid nucleus such as is typical of testosterone, progesterone and the adrenal cortical hormones. The finding that the capacity of testicular homogenates to convert Δ^5-androstene-3β, 17β-diol into α, β-unsaturated ketone was enhanced by estrogen pretreatment which concomitantly effected a marked curtailment in testicular androgen production is pertinent to the general concept as stated above. The data presented indicate that the reaction catalyzed by 3β-ol-dehydrogenase is not the limiting step accounting for the marked interference in androgen production as the result of estrogen administration. From our studies the failure of hormonal synthesis would appear to occur at some step other than that which involves the formation of the α, β-unsaturated ketone precursors. A similar conclusion was reached by Huseby, Samuels and Helmreich(5) from studies correlating the 3β-ol-dehydrogenase activity and androgen producing capacity of estrogen-induced interstitial cell tumors.

The relative quantitative differences between DESDP and estradiol-17β in effectiveness in disrupting androgen production and on stimulating the 3β-ol-dehydrogenase activity of testicular homogenates should be pointed out. From this study it can be concluded that estradiol-17β is considerably more active than DESDP in accelerating the rate of α, β-unsaturated ketone formation by testicular homogenate. Whereas it would appear from the DESDP pellet implant experiments that a near 2-fold increase in 3β-ol-dehydrogenase activity is the maximal stimulating effect capable for this estrogen, it was observed that estradiol-17β evoked nearly a 4-fold increase in testicular enzyme activity at 32 μg/kg/day. Although the data do not permit an accurate estimate of relative potency, it was observed that estradiol-17β at 2 μg/kg exhibited a somewhat greater effect on the tissue enzyme content than did DESDP at 114 μg/kg. It would therefore appear that the natural estrogen is at least 50 times more active than the synthetic estrogen in respect to elevating tissue content of 3β-ol-dehydrogenase.

These data do not answer the question as to whether or not the estrogen effect on the enzyme is independent of the pituitary. The atrophy of the sex accessory organs of the estrogenized rats indicates a decreased output of pituitary gonadotrophin, which from the report of Samuels and Helmreich(6) would be expected to bring about a lowering of the testicular enzyme content. These authors observed that the 3β-ol-dehydrogenase activity of testicular tissue fell well below normal values within 3-5 days after hypophysectomy. The administration of 100 I.U. of chorionic gonadotrophin per day restored the enzyme levels to values more than double those of intact control rats. These findings provide some basis for eliminating the possibility that the estrogen-stimulating effect on the testicular 3β-ol-dehydrogenase activity is a result of the inhibitory influence of estrogen on pituitary gonadotrophic function.

The possibility exists that the estrogen-stimulating effect is directly on the 3β-ol-dehydrogenase system. Thus, Gordon and Villee(7) have shown that the rate of DPN reduction in an isocitric dehydrogenase system isolated from placental extracts is a function of the concentration of estradiol-17β. The reduced DPN was measured spectrophotometrically at 340 mμ. In their experiments estradiol-17β was approximately 1000 times more active than diethylstilbestrol. Estrone was as active as estradiol, but none of a number of other steroids tested possessed this activity. Talalay and Dobson(8) demonstrated that estradiol-17β, but not estradiol-17α, possessed a strong protective effect upon 3β-ol-dehydrogenase isolated and purified from cultures of certain species of *Pseudomonas*. Whether or not the stimulatory influence upon testicular 3β-ol-dehydrogenase activity brought about by estrogen pretreatment as reported here is a manifestation of these suggested mechanisms remains to be determined.

Summary. 1. Pretreatment doses of estrogens, which brought about marked curtailment in testicular androgen production as evidenced by sex accessory organ atrophy, increased the 3β-ol-dehydrogenase content of rat testicular homogenates. Estradiol-17β was approximately 50 times more active than diethylstilbestrol dipalmitate in this respect. 2. This evidence suggests that the reaction catalyzed by the 3β-ol-dehydrogenase system is not the limiting one with respect to disrupted androgen production induced by estrogen pretreatment.

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Toxicity of Hydroxamic Acid Analogues; Prophylactic and Therapeutic Efficacy Against Nerve Gas Poisoning in Mice.* (22575)

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Hydroxylamine HCl and a series of its analogues were shown to accelerate the hydrolysis of phosphoric acid esters by splitting off the phosphate groups(1). Some of the hydroxamic acid derivatives (HA) are capable also of reactivating cholinesterase that had been inhibited by reaction with phosphoric acid esters, such as nerve gases and certain of the insecticides. This is accomplished by releasing the phosphate bound in the reaction, thus freeing the enzyme(2-6).

These evidences of potential prophylactic and therapeutic use of HA compounds were tested in mice intoxicated with nerve gas or with phosphate insecticides(7). Only studies with the nerve gas, Sarin (isopropyl-methyl-phosphono-fluoride), are reported here.

Method. Hydroxylamine and analogues of hydroxamic acid (HA) were tested both for their individual toxicities and for their efficacies in reducing the toxicity of Sarin in albino mice. Each HA was made up as a solution in 0.85% NaCl, neutralized and adjusted so that 1 ml contained approximately one LD_{50} when administered intraperitoneally. For each analogue groups of 6 to 8 mice were injected with 4 to 6 different doses covering a range of approximately the interval LD_1-LD_{100}. The LD_{50} for each analogue was calculated by the method of Litchfield and Wilcoxon(8). These are listed in Table I. Mice generally died within one hour. A few succumbed during the second hour and those that survived for 2 hours seldom died during the subsequent 18 hours of observation. Only deaths within 2 hours were used in the calculation of the LD_{50}. Animals were observed

TABLE I. Approximate LD_{50}'s in Mice of An Analogous Series of Hydroxamates Injected Intraperitoneally.

Hydroxamate	Approximate LD_{50} (mg/g)
Hydroxylamine	.15
Salicyl-	.15
Nicotin- (benzobromide)	.16*
Polyacryl-	.16
Diglycolic di-	.17*
Picolin-	.3
Tartar-	.5
Sorb-	.7
Glycin-	.8
Acet-	1.3
Nicotin-	2.3
n-dimethyl glycin- (methochloride)	2.5 *
Glucuron-	3.0
Histidin-	3.7
Nicotin- (methiodide)	4.0
Serin-	6.5
Lact-	8.4
Mandel-	>5.0 †

* Tested against 2 × LD_{50} only (Table II).
† Limited by solubility.

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* Synthesis of hydroxamates by Drs. T. Wagner-Jauregg, G. M. Steinberg and R. Plapinger, Medical Laboratories, Army Chemical Center, Md. Statistical analysis was done by Mr. I. A. DeArman, Medical Laboratories, Army Chemical Center, Md.

TABLE II. Effectiveness of Hydroxamic Acid Analogues, Given Intraperitoneally, against One or Two LD_{50}'s of Sarin Given Subcutaneously in Mice.

Hydroxamate	Min. from nerve gas inj.	Dose, mg/g	Potency ratio†	No. dead /No.inj.‡
Glycin-	–15	.4	1.5	
	–10	.4	1.6*	
	– 2	.4	2.4*	
	2.5	.5	2.5*	
	10	.5	2.1*	
	12	.5	1.5	
Lact-	– 5	.6	2.8*	
	– 5	.04	1.0	
	5	2.0	2.8*	
	5	.4	.8	
Nicotin-	– 5	.9	2.1*	
	– 5	.5	2.2*	
	5	1.7	2.9*	
Nicotin- (methiodide)	– 5	2.0	2.2*	
	– 5	.4	1.2	
	5	2.0	2.9*	
	5	.4	1.0	
Picolin-	– 5	.2	1.0	
	5	.2	.7	
	1-2	.2	1.0	
Serin-	– 5	1.5	1.4	
	– 5	1.5	2.7*	
Citric-	– 5	.4	1.3	
Sorb-	– 5	.2	1.5	
Polyacryl-	– 5	.4	1.0	
	– 5	.75	1.0	
Glucuron-	– 5	1.0	1.4	
n-dimethyl glycin- (methochloride)	– 5	1.0		8/24
Nicotin- (benzobromide)	– 5	.8		11/24
Diglycolic di-	– 5	.8		22/24

* Significantly > 1.0.

† Calculated LD_{50} compared to that of control mice.

‡ Against $2 \times LD_{50}$ ($> LD_{100}$) Sarin.

also for clinical evidence of toxicity as indicated by appearance and behavior with all doses for the purpose of choosing an initial trial protective dose.

Next the analogues were tested for protective efficacy in mice poisoned subcutaneously with one LD_{50} of Sarin. In order to control possible variability in the toxicity of Sarin the LD_{50} was estimated each time that an HA was to be tested against it. This was done by injecting 4 to 6 groups of 6 mice each with different doses of Sarin(8). On the same day 2 groups of 24 mice each were injected with the LD_{50} of Sarin subcutaneously. Of these groups one was injected intraperitoneally with HA also and the other with saline. The protective doses chosen for each HA used, listed in Table II, range up to ⅔ of the LD_{50} of the HA, the largest doses given being smaller than those provoking signs of toxicity. HA was given either before or after Sarin. (The terms "therapeutic" and "prophylactic" refer only to the time relationship with administration of Sarin rather than to mechanism.) The intervals to or from the time of injection of Sarin are listed in Table II. To assess the efficacy of an HA for each experimental situation, the numbers of dead out of 24 in the 2 groups were examined and the difference tested by means of χ^2 in a 2x2 contingency table(9). This is equivalent to testing the equality of the proportion dead in the 2 groups. The index chosen to measure the relative efficacy of an HA is the potency ratio (10). This was computed by pooling the results from all preliminary assays of Sarin, and computing a slope. It was then assumed that the slopes of the response lines of the experiments with HA would be equal to this common slope for all experiments. Using the common slope and the responses observed in each of the efficacy trials of HA against a relatively standard dose of Sarin, LD_{50}'s were estimated for each trial. Potency ratios were computed and appear in Table II. The significance of the potency ratio was determined by the χ^2 test. If the numbers of dead in the 2 groups were significantly different, the ratio was assumed to be significantly different from 1. In addition 3 HA's were tested against twice the LD_{50} of Sarin, a dose that kills all mice, and the results are shown at the end of Table II.

Results. Toxicity levels for the analogous series of hydroxamates, administered intraperitoneally, are listed in descending order (Table I) and indicate a wide range among those tested. It is of special interest that the potency ratios shown in Table II appear to be independent of the relative toxicities. For example, lact-hydroxamate is the least toxic and one of the more effective compounds. On the other hand, glycin-hydroxamate is more toxic and quite ineffective. The last 3 compounds listed in Table II protected all mice against one LD_{50} of Sarin and gave partial protection against 2, a uniformly fatal dose. Thus far, compounds having protective properties when injected several minutes prior to Sarin may also be effective when administered within a few minutes after the toxic material (Table II). "Prophylactic" or early "therapeutic" use of the hydroxamates intraperitoneally appears to be more effective, however, than their use subsequently. When administered after the onset of obvious signs of intoxication, the effects of these hydroxamates were negligible in terms of reversing the developing toxic manifestations in mice.

Discussion. Although there are striking hydrolytic effects of hydroxamic acid analogues *in vitro*(1-6), protection requires relatively huge doses in mice. This is of practical importance in application. Factors pertaining to optimum conditions for contact between the antidote and either free Sarin or Sarin-inhibited enzymes or both, *in vivo,* include rates of absorption and excretion, of degradation and detoxification and of diffusion from the circulation. The apparent independence of toxicity and potency ratios among candidate compounds encourages further exploration. It would appear that toxicity of an HA may limit the prospect of testing its maximum efficacy. However, the large doses required to raise the LD_{50} of Sarin significantly would appear to limit the therapeutic potential. Related compounds are being studied.

Summary. Some hydroxamic acid analogues protect mice against an LD_{50} dose of Sarin. Effective doses required in this series have been large. The efficacy of hydroxamates is variable and unrelated to toxicity.

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Host Resistance to Bacteria in Hemorrhagic Shock. VI. Effect of Endotoxin on Antibacterial Defense.* (22576)

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Transfusion for experimental hemorrhagic shock of 2 hours duration is followed by recovery. But if the shock is allowed to persist for much longer (4-5 hours) it fails to respond to transfusion. Evidence has been given that the failure of the circulation to respond to transfusion is a result of bacterial activity, which cannot be controlled because shock produces a progressively severe injury to the antibacterial mechanisms(1-6). The further observation that intravenous antibiotic therapy, given shortly after shock was induced, failed to prevent the development of irreversibility, in contrast to the success of such therapy when given before shock was induced

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TABLE I. Influence of Time on the Lethality of *E. coli* Endotoxin I.V. in Normal Rabbits.*

		Second inj. at time intervals stated below					
		10 min.		1 hr		4 hr	
First inj.	Second inj.	Total	% survival	Total	% survival	Total	% survival
MSD							
1	—	25	100	25	100	25	100
1/2	1/2	10	80	10	20	15	60
1/2	1/4	10	60	10	50	10	80
1/2	1/8	10	100	10	40	10	90
1/2	1/16	—	—	10	20	10	90
1/2	1/32	—	—	10	40	10	100
1/2	1/64	—	—	10	40	—	—
1/2	1/128	—	—	10	20	—	—
1/2	1/256	—	—	10	80	—	—
1/2	1/512	—	—	10	0	—	—

* MLD 100 = 0.13 mg/kg; MSD 100 = 0.05 mg/kg.

(3),[†] suggested that a process already in operation during the short interval between onset of shock and administration of the antibiotic was responsible for the development of irreversibility. This process was assumed to be the production of a small amount of bacterial endotoxin,[‡] which eventually caused failure of transfusion because of progressive collapse of the antiendotoxic defense(3). These considerations led to experiments designed to explore (1) the relationship of time to the efficiency of the mechanisms involved in the detoxification of endotoxins; (2) the effect of antibiotics on the response to endotoxins.

Method. A purified *E. coli* endotoxin[§] given intravenously was employed in all the experiments to be described. The intravenous MLD/100 (minimal lethal dose for all recipients) was 0.13 mg/kg and the intravenous MSD/100 (maximal surviving dose for all recipients) was 0.05 mg/kg for normal rabbits(6). One half MSD/100 was administered to each of a group of normal rabbits. Ten minutes later a second dose was given. The second dose, beginning with ½ MSD/100, was decreased in each successive rabbit by 50% until no effect was secured. This experiment was repeated in 2 additional groups of normal rabbits, except that the interval between the first and second dose was lengthened to one hour in one group, and to 4 hours in another group.

Results. Table I shows first, that 11% (6 of 55) of normal rabbits were killed by the second dose of toxin given 10 minutes after the first dose; and secondly, that 67% (47 of 70) were killed when the second dose was given one hour after the first safe dose. Even with a second dose as little as 1/128 of MSD/100, most of the recipients died. When the second dose was given 4 hours after the first, the mortality rate was 18% (10 of 55 rabbits). The survival time of most of these recipients was about the same (3-24 hours) as of those receiving an MLD/100. Some that survived showed varying degrees of transient

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[†] In our original experiments (1952) on dogs, penicillin, neomycin and the tetracyclines were effective when given prior to shock. In 1955 they were ineffective, except when given to dogs just prior to delivery to our laboratory from the dealer's kennels, and promptly submitted to the shock procedure, *i.e.*, before they could acquire the now resistant intestinal flora in our animal quarters. Prophylactic antibiotic therapy was, however, successful in rat experiments performed in 1955(7). This latter observation has been confirmed by Shorr and Baez(8).

[‡] Evidence has been given for the presence of bacterial activity in the tissues of dogs in hemorrhagic shock, in spite of the absence of positive blood or tissue cultures (except Clostridia) until a few minutes after death, and that this bacterial activity is responsible for development of irreversibility to transfusion(1). There is also evidence that bacterial invasion from the intestine is a continuous process in the normal as well as the shocked animal(7,9,10). Moreover there is reason to believe that endotoxins can be absorbed from the intestinal canal(6). Recently a leucotoxin which is neutralized by Properdin has been identified in the blood of the rabbit in hemorrhagic shock(11).

[§] We are grateful to Dr. A. Braude of the University of Texas Medical School for a generous supply of this material.

TABLE II. Effect of Preloading the RE System with Trypan Blue* on Toxicity of *E. coli* Endotoxin.

Normal rabbits			Hemorrhagic shock rabbits†		
Dose of endotoxin I.V. (mg)‡	No. rabbits	No. survived	Dose of endotoxin I.V. (mg)§	No. rabbits	No. survived
.05	1	0	.00000012	1	0
.025	4	0	.00000006	3	0
.0125	5	0	.00000003	5	1
.0063	5	1	.000000015	4	3
.0032	5	5	.000000007	3	3

* 10 cc of an 0.1% solution 3 times a wk for 7 wk.

† Hemorrhagic shock of 2 hr duration, produced by a technic described elsewhere (Schweinburg, F. B., Frank, H. A. and Fine, J., *Am. J. Physiol.*, 1954, v179, 523; and Schweinburg, F. B. and Fine, J., PROC. SOC. EXP. BIOL. & MED., 1955, v88, 589).

‡ .05 mg was upper limit of a uniformly non-lethal dose of a standardized *E. coli* endotoxin in normal rabbits.

§ Endotoxin given 4 hr after transfusion. .00000012 mg was upper limit of a uniformly non-lethal dose of a standardized *E. coli* endotoxin when given to rabbits 4 hr after transfusion for hemorrhagic shock of 2 hr duration.

muscular weakness and anorexia. Those that died showed progressive muscular weakness beginning within a few hours. They developed progressive hypotension, oliguria or anuria, and pallor of the mucous membranes. Gross post-mortem examination disclosed nothing more than scattered petechial hemorrhages, and a small amount of blood in the gut. There was no evidence of the Shwartzman reaction.

Comment. These results demonstrate that a small dose of endotoxin quickly induces a hypersensitivity to more endotoxin, but that substantial recovery of resistance to endotoxin is already present 4 hours after the initial dose. This is the interval required for blood clearance of a dose of endotoxin injected intravenously, and for maximal uptake by the RE system(12). That the RE system inactivates endotoxin is indicated by the observation (Table II) that the sensitivity of rabbits preloaded with Trypan Blue was much higher than in comparable rabbits not given the dye. Since shock injures the RE system(13), failure of detoxification can explain a persisting and even an increasing hypersensitivity to endotoxin during shock. That the death resulting from the second dose of endotoxin is due to the release and exposure to still more endotoxin is shown by the results of experiments (Table III) in which antibiotics (100,000 units of penicillin and 50 mg of Streptomycin given intravenously) were administered with the initial dose of endotoxin. In this situation, the second dose, given one hour later, proved to be virtually harmless, for the mortality rate fell from 67% to 4% (2 of 46 animals). Since the antibiotics protect by preventing the generation of more endotoxin‖, one might expect the

TABLE III. Coli Endotoxin I.V. Penicillin, streptomycin I.V. with first dose of endotoxin. Second inj. of endotoxin one hr later.

1st inj. of endotoxin	2nd inj. of endotoxin	No. of rabbits		
MSD		Total	Dead	Survived
1	—	25	—	25
1/2	1/2	10	1	9
1/2	1/4	10	0	10
1/2	1/8	5	0	5
1/2	1/16	6	0	6
1/2	1/32	5	0	5
1/2	1/64	5	0	5
1/2	1/128	5	1 (72 hr)	4
1/2	1/256			

MSD 100 = 0.05 mg/kg.

‖ The protective action of antibiotics in shock or against endotoxin cannot be due to other than their antibiotic properties for several reasons: 1. Penicillin has no known pharmacologic activity apart from its antibiotic action. 2. Except for the acceleration of growth from long-term administration in food, these compounds, in so far as they do exert any non-antibacterial properties, act adversely rather than protectively. 3. As will be shown below, Bacitracin and Polymyxin B, when given orally, protect even though they are wholly non-absorbable. Finally, it is improbable that such widely diverse chemical compounds could exert a uniformly protective non-antibacterial pharmacologic effect.

TABLE IV. Effect of Antibiotic Therapy on Survival Rate of Normal and Shocked Rabbits Subjected to Their Respective MLDs (100) of Bacterial Toxin or Multiples Thereof.

		Toxin		Normal		Shock	
Exp. No.	Antibiotic*	Dosage (MLD)	Time relative to antibiotic therapy	No. of rabbits	Survival (%)	No. of rabbits	Survival (%)
	None	1	—	20	0	20	0
I	Pen & S	1	4 hr after	10	60	20	45
		1½	”	10	50	20	50
		2	”	10	50	10	50
II	”	1	Simultaneously	20	55	20	45
		1½	”	10	60	20	45
		2	”	20	60	10	50
		3	”	10	0	—	—
III	”	1	”	20	65	10	40
		1½	”	20	60	10	60
		2	”	20	60	10	50
IV	B & P or N	1	Immediately after 4th dose or 6 hr later in 2 hr shocked dog	10	50	15	60
		1½	*Idem*	10	50	15	35
		2	”	10	50	10	40

* Pen = penicillin; S = streptomycin; B = bacitracin; P = polymyxin; N = neomycin.

timing of the antibiotics to condition the results. To determine the effect of timing and to disclose the source of the additional endotoxin, the following experiments were undertaken.

Method. Rabbits of the same breed and approximate weight (2-3 kg) were variously exposed to the *E. coli* endotoxin and to antibiotics as follows: *Group I*: Penicillin G. 100,000 U and Streptomycin (50 mg) were given intravenously to normal rabbits, and to rabbits immediately after transfusion for hemorrhagic shock of 2 hours duration. Four hours later one MLD/100 of endotoxin was administered intravenously to each rabbit in both groups. This dose, as stated, was 10^{-1} mg for normal rabbits. For rabbits 4 hours after transfusion for hemorrhagic shock of 2 hours duration the MLD/100 was 10^{-6} mg (6). The experiment was repeated with multiples of the respective MLDs/100. *Group II*: The procedure was the same as in Group I, except that the antibiotics were injected into one ear vein at the time the endotoxin was injected into the other. *Group III*: The procedure was the same as in Group II, except that the antibiotics and the endotoxin were injected together, after mixing and standing at 4°C for 4 hours. *Group IV*: Bacitracin (10,000 U) and Polymyxin (100,000 U) or Neomycin (100,000 μg) were fed daily by gavage for 4 successive days to 2 groups of normal rabbits. About one hour after the fourth dose each rabbit in one group received one MLD/100 endotoxin (10^{-1} mg) intravenously. The rabbits in the other group were put into hemorrhagic shock for 2 hours, and then transfused. Four hours later, each of these received one MLD/100 endotoxin (10^{-6} mg). *Group V*: Normal rabbits were given one MLD/100 of endotoxin, and the antibiotics employed in Group I were given 30, 60, or 90 minutes *afterward* instead of before or simultaneous with the endotoxin. All animals were observed until they were fully recovered, or until death, in which case a gross postmortem examination was made.

Results (Tables IV and V). Without anti-

TABLE V. Penicillin (100,000 Units) and Streptomycin (50 mg) I.V. Simultaneous with or at Various Intervals after a Single MLD/100 Dose of *E. coli* Endotoxin.

	No. normal rabbits	% survived
Simultaneously	50	50
10 min. after endotoxin	10	10
30 ” ” ”	10	0
1 hr ” ”	6	0
1½ ” ” ”	4	0
2 ” ” ”	6	0

biotic therapy the expected survival rate of both the normal and shocked rabbits would have been 0%(6). The average survival rate proved to be about 50% in Groups I, II, III, and IV, *i.e.*, in those in which antibiotics were given *prior to or simultaneous with* the administration of endotoxin (Table IV). In Group V, in which the antibiotic was given 30 or more minutes *after* the endotoxin, the survival rate was 0% (Table V).

Comment. The results of the Group I, II, and III experiments confirm those of numerous investigators(14-19), who noted a considerable decrease in the lethal power of certain bacterial toxins as a result of prior administration of certain sulfonamides or antibiotics. No explicit view of the way these agents affect the action of toxin is offered in most of these reports, but several investigators(18,19) assert that the antibiotics exert an anti-endotoxic action *in vivo*. Our Group III experiments exclude a direct effect of the antibiotics on the endotoxin. Our Group IV experiments, in which non-absorbable antibiotics (Bacitracin and Polymyxin B) introduced into the intestine were as effective as absorbable antibiotics given by the same route (Neomycin¶) or intravenously, exclude both a direct and an indirect effect upon the endotoxin. Therefore, the antibiotics act only by suppressing bacterial activity. The Group IV experiments demonstrate further that the bacteria which are the source of the additional toxin are to an important degree, if not entirely, those invading from the gastrointestinal tract.

It follows that a lethal dose of test endotoxin is not necessarily lethal *per se*, and that death involves an effect consequent to injury by the endotoxin to the antibacterial defense mechanisms, with the subsequent elaboration of more bacterial toxin. This additional dose, added to the test dose, constitutes the lethal dose. From the results of the experiments with multiples of the MLD/100 it would appear that the additional dose may equal as much as 1 MLD/100 of the test endotoxin, because the antibiotics achieved the same degree of protection with test doses up to 2 MLD/100. In the first 4 groups of experiments the antibiotics were immediately available to shut off the generation of more endotoxin than was administered to the animal. In the Group V experiments the antibiotics were not immediately available, and proved completely ineffective. The effect of this difference in timing is strikingly analogous to the effect of the difference in timing of antibiotic therapy in hemorrhagic shock. We have attributed the failure of antibiotic therapy applied soon *after* the induction of hemorrhagic shock, in contrast to its effectiveness if applied *prior* to shock, to the interim production of a lethal dose of bacterial toxin (3). This assumes that the antibacterial functions begin to fail very shortly after the induction of shock, and that the toxin which can develop in the interval between the onset of shock and the administration of antibiotic constitutes a lethal dose eventually, because of the rapid and enormous rise in sensitivity to the toxin, which cannot be neutralized or excreted. The results of the Group V experiments, as contrasted to those of Groups I-IV, suggest that a similar process operates when a lethal dose of endotoxin is given; *i.e.* that the antibacterial defense mechanisms are as promptly and as severely damaged by a large dose of endotoxin as by hemorrhagic shock, and that exposure to an otherwise insignificant amount of endotoxin shortly thereafter is sufficient to produce irreversible shock as a result of increasing sensitivity to endotoxin. Presumably a dose of endotoxin is lethal because it permanently paralyzes the recuperative powers of the detoxifying system, whereas a sublethal dose does not prevent their recovery after a certain interval unless an additional, though relatively trivial, dose is added in the interim.

This investigation was done with one endotoxin only. The results obtained may not hold for other endotoxins. But because there are no identifiable distinguishing features among the various endotoxins, we may assume that their pathological effects are similar.

Summary and conclusions. 1. The mortality rate from one intravenous MLD/100 of endotoxin was reduced from 100% to 40-60% by antibiotic therapy given prior to or at the time of injecting the endotoxin. This was true even if the antibiotic was given orally and was completely non-absorbable. Therefore, endotoxins from intraintestinal bacteria are involved in the death from an intravenous MLD/100 of endotoxin. Data are given to show that antibiotics act solely as antibacterial agents, and not as anti-endotoxic agents. 2. A sublethal intravenous dose of endotoxin promptly induced in rabbits an increased sensitivity to a supplementary fractional dose of endotoxin so that a second dose given one hour later increased the mortality from zero to 67%, even though the total of both doses, if given as a single initial dose, was not lethal. Recovery of nearly normal resistance to the second dose of endotoxin required about 4 hours. Antibiotics given with the initial dose rendered the second dose harmless; but the same antibiotics given with the second dose did not prevent death. Therefore, it is inferred that death from the second dose involved the production of additional endotoxin. 3. Reasons are given for inferring that similar phenomena operate in hemorrhagic shock and account for the development of irreversibility to transfusion.

¶ A very small fraction of neomycin is absorbed.

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Serial Propagation of Adenoviruses (APC) in Monkey Kidney Tissue Cultures. (22577)

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Production of adenovirus (APC) vaccines suitable for general usage, is dependent on the serial propagation of virus in cultures of acceptable non-malignant tissue. Since monkey kidney tissue cultures demonstrated cytopathogenic effects and supported limited growth of adenoviruses(1), it seemed possible that with further adaptation, these agents might be induced to grow satisfactorily in such cultures. This report describes the procedures used in establishing monkey kidney passage lines of 7 serotypes (1 through 7) of adenoviruses. None of the strains was grown in HeLa cell or other cancer cell cultures at any point in its passage history.

Methods. Rhesus monkey kidney tissue cultures* prepared by the Youngner procedure(2) in stationary tubes and 32 oz. bottles were grown in 2.0% calf serum, 0.5% lactalbumin hydrolysate, and 97.5% Hanks' solution(3), and maintained in 1.0 and 40.0 ml respectively of Mixture 199(4). Bottle cultures were washed 3 times with 20.0 ml of Hanks'-Simms' solution prior to inoculation. Nutrient fluids were changed routinely twice a week for tube cultures and once a week for bottle cultures. Occasionally, only one-half of the bottle culture fluid was changed, and in advanced passages bottles were maintained without fluid change (usually 7 to 10 days).

Monkey kidney passage lines of types 1, 2, 5, and 6 were initiated with material from early passages in human embryo tissue of fluids from spontaneously degenerating adenoid or tonsil tissue cultures(5). Strains of types 3 and 7 were obtained by inoculating a number of virus-containing throat washings into monkey kidney cultures. Virus strains recovered from several such specimens were carried for one or two additional passages and the most rapidly cytopathogenic strain of each type selected for further passage. Attempts were made to initiate passage lines of type 4 by direct isolation in monkey kidney culture, but of 8 known-positive nasal washings tested, only one produced cytopathogenic changes, and this strain could not be passaged further. Therefore, this strain was reisolated in human embryo trachea tissue culture and then passaged to monkey kidney cultures. Initiation of passage lines and routine serial passages were carried out by the inoculation of 0.1 ml of sample into monkey kidney tubes containing 0.9 ml of Mixture 199; supernatant fluids harvested at the time of maximal tissue involvement were usually used for passages. If cytopathogenic effects were delayed in passage attempts, cultures were reinfected at the time of the first fluid change with pools of tissue scrapings and several fluid harvests of the preceding passage, or passages were repeated with 0.2 to 0.4 ml inoculum per tube.

Attempts at establishing the viruses in bottle cultures were made by inoculating 0.5 to 2.5 ml of supernatant fluids from infected tube cultures; the bottles were frequently reinfected with similar amounts of fluid if cytopathogenic effects were delayed or progressed slowly. The final fluid harvests and frequently intermediate fluids from bottle cultures were titrated as antigen in the complement-fixation test using a standard pool of human convalescent serums(1). Since in early passages replicate bottles occasionally varied as much as 16-fold in antigen yield, the fluids showing the highest CF antigen titers were selected for passage. Fluids of early bottle passages were centrifuged for 4 to 4½ hours at 30,000 rpm in a Spinco Model L centrifuge; the pellets were resuspended in the lower one-fifth of the supernates and 3.5 to 5.0 ml quantities used as inocula for subsequent passage. Serial passages were made with ultracentrifugal concentrates until harvests with high virus and CF antigen titers were regularly obtained.

Results. Serial passages of all 7 serotypes were established initially in tube cultures by selection of strains giving the shortest incubation periods and by the use of large inocula and reinfection of cultures. In the first kidney passage, cytopathogenic changes developed in 1 to 5 days, but in subsequent passages the incubation periods were usually markedly prolonged, and the virus and CF antigen yields were very poor. One or 2 bottle passages using concentrated inocula resulted in adaptation of types 1, 2, 5 and 6 to give satisfactory virus and CF antigen titers in further passages. On the other hand, 3 to 5 such passages of types 3, 4 and 7 were necessary before satisfactory harvests were obtained, and occasional subsequent passages with concentrated virus were necessary to maintain yields of virus having high titers (Table I). All 7 serotypes were carried through 10 or more serial passages in monkey kidney cultures. Infectivity titers for HeLa cells(1) at 4th to 9th passage levels of types 1, 2, 3, 5, 6, and 7 were $10^{4.5}$ to 10^6 TC50

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* Cultures were obtained from Microbiological Associates, Bethesda, Md.

TABLE I. Representative Serial Passage Lines of Adenoviruses in Monkey Kidney Tissue Cultures.

Type	Passage	Type of culture	Vol of inoculum per culture, ml	Days to 1st cytopathogenic effect	CF titer of harvest (highest level
3	MK* 1	Tube	.1‡	5	
	2	”	.1	6	
	3	”	.1	2	
	4	Bottle	1.0	4	1:1
	5	”	5.0 5× concentrate	5	1:1
	6	”	3.5 ”	3	1:16
	7	”	1.0	5	1:16
	8	”	2.0	3	1:4
	9	”	3.5 ”	<6	1:16
	10	”	2.0	2	1:16
4	HE Tr† 1	Tube	.1§	6	
	MK 1	”	.1	1	
	2	Bottle	2.0	10	1:4
	3	”	5.0	5	1:1
	4	”	4.0 5× concentrate	5	1:4
	5	”	4.0 ”	4	1:4
	6	”	4.0 ”	2	1:8
	7	”	4.0 ”	1	1:32
	8	”	1.0	2	1:32
	9	Tube	.01	1	
	10	”	.1	1	

*Monkey kidney. † Human embryo trachea. ‡ Throat wash J.F. § Nasal wash R.N.

per 0.1 ml, representing virus activity at theoretical cumulative dilutions of the original inocula of 10^{-15} to 10^{-19}. After 8 passages, type 4 titrated $10^{3.5}$ or higher, when the cumulative dilution of inoculum was 10^{-23}. After 6 to 10 passages, all types produced CF antigen titers of 1:16 or greater in 2 or more bottle harvests. The identities of the passage viruses were confirmed in neutralization tests performed in monkey kidney cells; all strains were completely neutralized by 8 units of homologous rabbit antiserum. Since the rabbit antiserums had been prepared with viruses grown in HeLa cells or human embryo tissue and with no history of passage in monkey kidney cells, these tests also indicated that the passage lines were not contaminated with monkey renal viruses(6). Formalinized and heat-inactivated vaccines prepared with the monkey kidney passage line of type 3 stimulated neutralizing antibody responses and prevented an experimentally induced illness in volunteers(7). Similarly, a formalinized polyvalent vaccine containing the monkey kidney passage strains of types 3, 4, and 7 induced neutralizing antibody responses to all 3 serotypes in rabbits and humans, and reduced febrile respiratory disease rates in Navy recruits(8).

Summary. Strains of adenovirus types 1 through 7 were carried through at least 10 serial passages in monkey kidney tissue cultures, yielding harvests of virus with titers of $>10^{3.5}$ to 10^6 per 0.1 ml, and frequently CF antigen titers of 1:16 or higher.

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Further Studies with Cell-Free Extracts from Mouse Spleen on X-Ray Induced Mortality.*† (22578)

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We have been able to offer evidence(1) in support of a humoral spleen factor effective in the recovery of mice from X-ray-induced mortality. In these studies it was shown that administration of cell-free saline extracts from spleens removed 6 days after exposure of the donor mice to the LD 30/14 days of X rays resulted in a definite and statistically significant reduction of mortality. However, extracts made from spleens of donor animals exposed to the LD 55/14 days and removed at the same time after irradiation failed to show such protective effect. The results obtained with extracts from normal spleens were ambiguous. The ambiguity was partly traceable to a saline effect on X-ray mortality previously described(2).

Studies in which it was possible also to demonstrate the presence of the humoral spleen factor in extracts from normal mouse spleen tissue are here presented.

Methods. A. *Experimental Animals.* Male white Swiss mice of the Institute stock, weighing 25 ± 4 g were used. B. *Irradiation* of the mice took place as previously described in groups of 12 animals(3). The radiation factors were 200 KVP, 25 ma, 0.25 mm Cu + 1.0 mm Al added filter, HVL = 0.8 mm Cu. The X-ray equipment was calibrated prior to each set of exposures.‡ All animals to be used for the evaluation of spleen extracts were exposed to 425 r/air which dose represents, under the conditions of this experiment, the LD 78/15 days. (Table I, group A.) C. *Spleen Extracts.* Unirradiated or irradiated mice were killed by dislocation of the neck and the spleens removed immediately. The organs were quick-frozen with dry ice, ground to a fine powder in a mortar cooled with dry ice. Various amounts of 0.9% saline were added resulting in concentrations of one, two, etc., spleens per cc. The mixture remained for extraction in a refrigerator for at least 24 hours and was then centrifuged at 3-5°C at 2,500 rpm for 30 minutes for the removal of grosparticulate matter. The supernatant was decanted into a sterile beaker and passed through a Buchner filter with several layers of filter paper under light vacuum. This filtrate was then passed through a Selas filter of 03 porosity under heavy vacuum, to obtain extracts free from viable cells and bacterial contamination. The extracts were injected intramuscularly in amounts of 0.3 cc daily for 5 consecutive days starting on zero day immediately after irradiation. D. *Control of reproducibility of extraction method* took place by means of micro-Kjeldahl determinations of total protein and NPN content of the extracts, using a standard distillation method(4). E. *Recording and presentation of effects of extracts on X-ray-induced mortality.* The number of dead animals in each experimental group was recorded daily over a period of 20 days. The following groups were used: (a) non-medicated, irradiated animals; (b) saline-treated, irradiated animals; (c) spleen extract-treated, irradiated animals. F. *Evaluation of results.* The number of survivors in the spleen-treated and saline control groups of equal sizes was compared on the 20th postirradiation day. The time of comparison has been selected on the basis of our preliminary studies(1) showing that an effect of spleen extract was not noticeable prior to the end of the second postirradiation week, and that no important changes in mortality rates occurred after this date. This choice was furthermore determined by our observation that at the 20th

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* The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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‡ We are indebted to CDR J. E. Morgan, MSC, USN, for these calibrations.

TABLE I. Comparison of X-Ray-Induced Mortality in (A) Non-Medicated, Irradiated Mice; (B) Saline-Treated, Irradiated Mice; (C) Normal Spleen Extract-Treated, Irradiated Mice.

Days after irradiation →	Group A Non-medicated					Group B Saline controls					Group C Spleen extracts				
	0	5	10	15	20	0	5	10	15	20	0	5	10	15	20
											Extr D				
Exp. 1	72*	72	63	14	11	72	71	53	18	15	72	67	47	24	22
											Extr 2D				
2	48	48	36	13	9	48	48	30	7	5	48	48	32	13	12
											Extr 4D				
3	24	24	16	4	3	24	24	21	3	3	24	24	23	5	5
Total animals alive	144	144	115	31	23	144	143	104	28	23	144	139	102	42	39
Mortality, %	0	0	20	78	83	0	1	28	80	83	0	3	29	70	73

* No. of survivors.

postirradiation day, 100% of the survivors of an exposure to 425 r/air were 2 ± 1 animal in 12 groups of 12 mice each. This consistency in the number of survivors among irradiated non-medicated controls appeared advantageous to the study of deviations in the number of survivors caused by medication of irradiated animals.

Results. 1. *Influence of saline injection on mortality produced by standard X-ray dose.* In view of the previously described saline effect on X-ray mortality(2), it appeared necessary to ascertain whether or not injection of a volume of saline equal to that of the spleen extracts would influence the X-ray-induced mortality under the conditions of the present experiment. Comparison of groups A and B of Table I shows that saline did not alter the X-ray-induced mortality significantly.

2. *Effect of extracts from unirradiated mouse spleens on X-ray mortality.* For evaluation of the effect of extracts made from normal mouse spleens, the following preparations were used: 1 spleen/cc, 2 spleens/cc, and 4 baby mouse[§] spleens per cc of saline, representing the extracts designated as D, 2D, and 4D respectively. The data obtained are summarized in column C of Table I (Exp 1-3). In each group there were more survivors in the spleen-treated group of animals than in an equally large group of simultaneously observed saline controls. While the differences in each of these groups are not of statistical significance, these observations indicate a definite trend toward higher survival in the spleen-treated animals. Comparison of total experience for groups B and C, comprising 144 spleen-treated and 144 saline controls, shows that there were 39 survivors among the spleen extract-treated animals and 23 among the saline controls. The difference between 39 and 23 is significant since $\chi^2 = 4.6246$; $P = 0.02$ to 0.05.

3. *Effect of extracts from spleens from irradiated donor animals on X-ray mortality.* Donor animals were exposed to the LD 55/14 days which, under the conditions of our experiment, was represented by 410 r/air. Spleens were removed on the 6th and 10th day after irradiation, respectively. The extracts were designated as $DX_6/410$ r and $DX_{10}/410$ r respectively, the letter D indicating that one spleen was extracted with 1 cc of saline; X stands for irradiation; the subscript indicates day of removal of spleens from the donor mice exposed to the X-ray dose shown after the slant. The results obtained with these extracts (Exp 4 and 5) are shown in Table II. No difference was found in the number of survivors of either of the 2 spleen-treated groups when compared with their respective saline controls.

4. *Reproducibility of spleen extraction method.* To ascertain the reproducibility of the spleen extracts a determination was made of total protein and NPN content of the extracts. The following averages were found: spleen weight 135 mg (120-160), total protein 975 mg % (710-1,290), and NPN con-

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[§] *i.e.*, mice 2 to 3 days of age.

TABLE II. Comparison of X-Ray-Induced Mortality in Saline-Treated, Irradiated Mice with That of Irradiated Mice Treated with Spleen Extracts from Irradiated Donor Animals.

	Group B Saline controls					Group C Spleen extracts				
Days after irrad. →	0	5	10	15	20	0	5	10	15	20
						Extr DX_6/410 r				
Exp. 4	48*	48	36	10	9	48	48	30	13	9
						Extr DX_{10}/410 r				
5	36	36	20	7	7	36	36	23	9	7
Total animals alive	84	84	56	17	16	84	84	53	22	16

* No. of survivors.

tent 54.5 mg % (41.6-70.0). These data indicate a reasonable reproducibility of this relatively crude extraction method. To obtain information on quantitative aspects of the extraction method, similar determinations were made in extracts where 2 spleens were extracted with 1 cc of saline. Two determinations of total protein and NPN in such extracts resulted in averages of 1,850 mg % of total protein (1,800-1,900), and 98.4 mg % of NPN (83.8-113.0). The chemical analysis thus indicates a satisfactory reproducibility of the extraction method also from the quantitative aspect. This view is furthermore supported by the data on baby mouse spleens. Average weight of the baby mouse spleens was only 18 mg, ⅛ the average weight of the adult mouse spleens.

Concentration of spleen in the preparation using 4 baby mouse spleens is about ½ that from 1 adult mouse spleen using 1 spleen/cc. Total protein content in the 3 determinations averaged 427 mg% (350-490) and NPN 30 mg % (29.0-31.7). These values are one-half of averages shown for D extracts and further indicate reproducibility of the extraction method. Finally data obtained from the study of extracts made with one spleen from an irradiated animal per cc are given. The average weight of these spleens was 44.7 mg (38-55), roughly ⅓ of the average weight of the unirradiated spleens. Average values for total protein and NPN were 253 mg% (220-270) and 12.9 mg % (5.1-17.2), respectively, about ¼ the average values found for normal spleens. The definitely lower total protein and NPN content of this preparation in contrast to the preceding ones is noteworthy.

Discussion. In this study the existence of a humoral factor in cell-free saline extracts made from spleens of unirradiated mice has been clearly demonstrated. These results round out our preliminary studies in which the presence of this factor was not demonstrated due to technical difficulties. It appears noteworthy that spleen extracts varying over a wide range in their protein and NPN content offer protection against X-ray doses which would otherwise kill about 78% of mice on the 15th day after irradiation. The significance of the data here presented appears to be enhanced by the fact that spleen extracts made from spleens removed from donor animals exposed to an X-ray dose killing about 55% of the animals within 14 days failed to show a protective effect, in line with our previous observations(1). The failure of these extracts to protect is paralleled by the results of the chemical studies of extracts made from these irradiated spleens which show a total protein and NPN content of about ¼ that found in equally prepared extracts from normal spleens, while spleen concentration amounted to roughly ⅓.

It would be tempting to assume that the spleens reduced in weight by irradiation to ⅓ of average weight of normal spleens are deficient in this humoral factor. However, such a statement could only be made with certainty if extracts made from irradiated spleens in concentrations equal to those made from normal mice would also show reduction in protective effect. Unfortunately, technical difficulties (irradiation of the very large numbers of donor mice necessary to obtain sufficiently large amounts of extracts) prevented the use of such concentrated extracts from irradiated spleens. The question therefore remains whether the differences between effects of extracts made from spleens of unirradiated and irradiated donor animals are merely due to a quantitative difference in concentration of the extractable factor, or whether the irradiated organ, shrunk to one-third of normal size and consisting chiefly of fibrous tissue, is functionally deficient with respect to elaboration of the humoral factor.

Our previous experiences(1), in which a protective spleen extract was obtained from organs of irradiated animals which were in an active stage of regeneration and reduced to only one-half or less of the average normal spleen size, support the last assumption.

Summary and conclusions. 1. Continuing previous studies, employing improved bioassay methods, the protective effect of cell-free saline extracts made from spleens of normal mice against X-ray-induced mortality in this species has been demonstrated. 2. Extracts made from the spleens of donor animals exposed to an X-ray dose which killed 55% of the donor animals in 14 days failed to show a protective effect, which was in agreement with previous observations. 3. A certain amount of parallelism between the protective effect of various extracts made from normal mouse spleens and the failure to protect of those made from irradiated mouse spleens was found in studies of the total protein and NPN content of such extracts. 4. It appears that within a wide range of concentration of total protein and NPN content such protection can be achieved. 5. However, reduction of average content of total protein and NPN below one-half of lowest average value for normal spleens, ascertained in samples made from spleens of irradiated donor animals, was found to be associated with a lack of protection. Whether this failure is due merely to quantitative or also to qualitative differences in these extracts is not clear.

Since the writing of this paper, an article by Allen *et al.* appeared in *Science,* 1956, v123, 1080, in which postirradiation protection of rabbits with plasma obtained from the splenic vein has been reported. Presence of a humoral spleen factor has thus been indicated in another animal species with a different method.

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Anaphylaxis in the Mouse: Possible Relation of the Schultz-Dale Reaction to Serotonin Release.* (22579)

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The evidence against the release of histamine in mouse anaphylaxis has been summarized(1). The purpose of the experiments reported here is to determine, using the Schultz-Dale technic, whether or not the substance responsible for contraction of the sensitized mouse uterus in the presence of antigen is serotonin (5-hydroxytryptamine).

Materials and methods. The virgin female mice were of the DBA or BALB/c strains, and were over 6 months of age when used. The mice were immunized intramuscularly in the hind legs with alum-precipitated egg white, as previously described(1). This technic of immunization is sufficient to render the mice anaphylactically sensitive to egg albumin as evidenced by the occurrence of a characteristic shock reaction on the intravenous injection of egg albumin(2); and by a contraction of the uterine smooth muscle of the sensitized mouse upon the addition of egg albumin *in vitro*, using the classical Schultz-Dale technic(1). Mice which had not received a prior injection of antigen never reacted by either technic when egg white was

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added. Further evidence of a real anaphylactic sensitivity was the observation of desensitization of the sensitized uterus after the addition of antigen(1). Circulating antibodies have been demonstrated in mice after immunization under parallel conditions(3); and the passive transfer of sensitivity has been effected by the administration of antiserum from similarly immunized mice to normal mice(1). Testing for intracutaneous sensitivity in the immunized mouse has repeatedly given negative results.

The technic used in the Schultz-Dale reaction, as previously described(1) consists, in brief, of attaching one-half of one uterine horn from the mouse to a lever in a water bath, and recording the contractions on a kymograph prior to and during the addition of the antigen.

Because this study is specifically intended to demonstrate drug inhibition of the anaphylactic reaction on *known* sensitized uteri, the criterion selected for determining sensitivity to egg white was the demonstration of a positive Schultz-Dale reaction on one or 2 of the 4 uterine strips. Only those mice were used which fulfilled this criterion. When normal mice were used to test drug sensitivity, it is so noted in the text.

Results. Reactivity of mouse uteri to serotonin. Serotonin[†] was dissolved in Ringer's solution, and the reactivity of normal and of sensitized uteri determined. It was found that a dose of 0.05γ/ml of bath was always sufficient to produce a good contraction in normal or sensitized uterine strips. Some strips reacted equally well to a smaller quantity. This amount is similar to that required to stimulate the mouse jejunum(4) and the rat uterus(4,5,6). *Inhibition of serotonin contraction with lysergic acid diethylamide (LSD) and with reserpine.* The addition of LSD[‡] in a concentration of 3γ/l was sufficient to prevent the reaction to 0.05-0.1γ/ml of serotonin when LSD was added immediately before the serotonin; 1γ/l was sufficient when the serotonin was added after 5 minutes had elapsed. These findings are comparable to those of previous workers(5-7) for the rat uterus. Reserpine[§] in a concentration of 5 mg/l was sufficient to antagonize the reaction to 0.05-0.1γ/ml of serotonin when in contact with the uterus for 5 minutes prior to addition of serotonin. This amount is 5 times greater than that reported recently by Costa(7) as being necessary to antagonize the serotonin contraction of the rat uterus; smaller concentrations were not tested here. *Inhibition of anaphylactic contraction with LSD and with reserpine.* A total of 16 sensitized mice were used: 6 were tested by showing positive reactions to egg white on 2 of the 4 uterine strips, and complete inhibition of anaphylaxis in the presence of 1-3γ/l of LSD on the other 2 strips; 6 were tested similarly, using 5 mg/l of reserpine; one strip from each of the remaining 4 mice was tested to demonstrate the anaphylactic contraction, one strip was tested in the presence of LSD, and one in reserpine. Following each test in the presence of drug, the reaction of the strip to serotonin and to 10γ/ml of acetylcholine(1) was tested. Uterine strips tested in the absence of drug gave uniformly positive Schultz-Dale reactions on the addition of egg albumin. In all of the 10 mice tested with LSD, 6 in duplicate, the reaction to egg albumin was completely inhibited; in all of the 10 with reserpine, identical inhibition was shown. The drugs inhibited the reaction to serotonin in every case, and never inhibited the reaction to acetylcholine.

Discussion. The evidence against the importance of histamine in mouse anaphylaxis consists primarily of the fact that the intact mouse and the isolated uterine strip of the mouse are sensitive only to enormous doses of the drug, that the body of the mouse probably does not contain sufficient histamine to produce a reaction, and that anaphylaxis in the intact mouse or on the isolated uterine strip cannot be inhibited by antihistaminic drugs.

[†] Serotonin creatinine sulfate, Nutritional Biochemicals Corp., Cleveland. Concentration calculated on weight as 5-hydroxytryptamine.

[‡] L.S.D.25, Sandoz Chemical Works, Inc., Hanover, N. J.

[§] Reserpine, Nutritional Biochemicals Corp., Cleveland. Dissolved in equal parts ethylene glycol and ethyl alcohol with 0.02% HCl 1-500 dilution in tissue bath.

It has been shown in this study that the isolated uterine strip is sensitive to 0.05γ/ml of serotonin, while a concentration of histamine of 50γ/ml(1) is necessary to produce a reaction. Thus the isolated tissue of the mouse is 1,000 times more sensitive to serotonin than it is to histamine. This sensitivity is completely abolished with the serotonin antagonists LSD and reserpine; and the anaphylactic contraction is completely inhibited in the presence of these drugs. Serotonin is the only substance known to be inhibited by these two drugs. It has also been shown(6) that the tissues of the mouse contain a quantity of serotonin easily sufficient to produce the anaphylactic response. These findings support the hypothesis that the anaphylactic reaction in the mouse is dependent on the release of serotonin.

Summary. (1) The egg white sensitized or normal mouse uterus is 1,000 times more sensitive to serotonin than it is to histamine. (2) This sensitivity is completely abolished in the presence of two known serotonin antagonists, LSD and reserpine. (3) LSD and reserpine, which as far as is known inhibit only serotonin, always and completely prevented the anaphylactic contraction on strips of demonstrably sensitized uterus in the ten trials with each drug.

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Teratogenic Effects of Herpes Simplex, Vaccinia, Influenza-A (NWS), and Distemper Virus Infections on Early Chick Embryos.*† (22580)

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The discovery of a causal relationship between maternal rubella and fetal pathology in human beings(1) has led to an experimental investigation of the role played by infectious agents in the production of congenital malformations. Recent studies concerning viral teratogenesis have resulted in the finding that characteristic developmental abnormalities and death follow infection of early chick embryos with the PR8 strain of influenza-A virus (2,3). Moreover, the induction of these teratogenic effects has been prevented by neutralization of the virus with specific antiserum, thus providing evidence that the virus was the cause of the syndrome(3). In addition, Newcastle disease virus has also been found to produce specific malformations in this embryonic host(4-6). In view of these results, experiments were conducted in this laboratory to determine whether the induction of teratogenic and lethal effects may be a property of other viruses as well. It is the purpose of this paper to describe the effects produced by infection of early chick embryos with herpes simplex, vaccinia, the NWS strain of influ-

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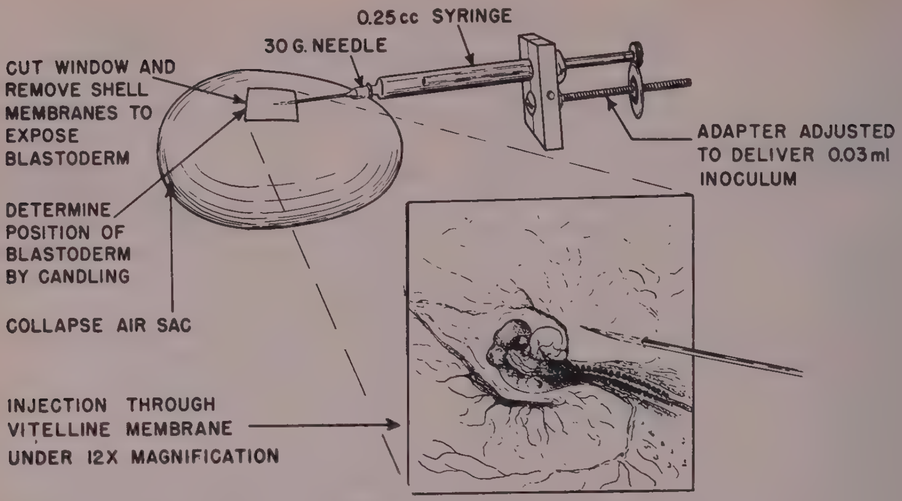
0.25cc SYRINGE
30 G. NEEDLE
CUT WINDOW AND REMOVE SHELL MEMBRANES TO EXPOSE BLASTODERM
ADAPTER ADJUSTED TO DELIVER 0.03 ml INOCULUM
DETERMINE POSITION OF BLASTODERM BY CANDLING
COLLAPSE AIR SAC
INJECTION THROUGH VITELLINE MEMBRANE UNDER 12X MAGNIFICATION

FIG. 1. Schematic presentation of method employed for inoculation of 48-hr chick embryos.

enza-A and distemper viruses.

Materials and methods. Inoculation of embryos. Embryonated hens' eggs, which had developed to stages 11-14 as described by Hamburger and Hamilton(7) after incubation at 37.5°C for 48 hours, served as the experimental hosts. The method of preparation and injection is shown schematically in Fig. 1. Following injection, eggs were reincubated and observed daily by temporarily removing the scotch tape "window" covering the site of inoculation. A more complete description of the inoculation procedure as well as the method of observation has been presented in a previous publication(3). *Viruses.* Three of the viruses investigated were obtained from 12-day-old embryonated eggs which had been infected 2 days earlier. Of these, the HF strain of herpes simplex virus and an egg-adapted strain of vaccinia virus were employed in the form of suspensions of triturated chorioallantoic (C-A) membranes, while infective allantoic fluid served as the source of the NWS strain of influenza-A virus. The herpes and NWS viruses had been maintained previously by intracerebral passage in mice. The fourth agent studied, an egg-adapted strain of distemper virus (Lederle), was prepared by trituration of C-A membranes obtained from 14-day-old embryonated eggs which had been infected 7 days previously. All viruses were maintained in frozen storage at —40°C. When required for use, the frozen material was permitted to thaw at room temperature and diluted with Difco Brain Heart Infusion (B.H.I.) medium containing 500 units of penicillin and 100 μg of streptomycin per ml. *Controls.* B.H.I. medium, a 10^{-4} concentration of uninfected allantoic fluid and a 20% suspension of uninfected triturated C-A membranes were employed as control inocula. All dilutions were prepared with B.H.I. medium to which antibiotics had been added. As an additional control, concentrations of virus known to uniformly produce anomalies and/or lesions (10^{-2} herpes, NWS, and vaccinia; 20% distemper) were subjected to heat exposure prior to inoculation of embryos. Heat inactivation was accomplished by placing tubes of virus in a water bath at 60°C for 1 hour. *Virus recovery.* To provide evidence that anomalies and/or lesions were associated with viral infection of the embryo, titration studies of embryonic pools were conducted. Accordingly, 4 groups of 48-hour embryos were inoculated with each of the viruses under study employing as inocula 10^{-4} concentrations of herpes, NWS, and vaccinia viruses and a 20% suspension of distemper virus. C-A membranes of 5 embryos infected with distemper virus were pooled 7 days after inoculation, while pools were made of 5 anomalous dead embryos within each of the remaining 3 groups 2-3

TABLE I. Incidence of Teratogenic and Lethal Effects upon Early Chick Embryos Produced by Infection with Herpes Simplex, Vaccinia, and Influenza-A (NWS) Viruses.

Virus and conc.	No. of embryos	Effects observed*	Days following inoculation					
			1	2	3	4	5	6-14
10^{-1} Herpes simplex	45	(A)	93	100				
		(D)	4	100				
10^{-2}	32	(A)	100					
		(D)	0	97	100			
10^{-3}	24	(A)	75	96	100			
		(D)	0	79	96	100		
10^{-4}	30	(A)	27	93	100			
		(D)	0	57	93	100		
10^{-5}	22	(A)	9	27	36	55	55	55
		(D)	0	9	23	64	86	86
10^{-6}	20	(A)	0	10	20	20	25	25
		(D)	0	0	15	25	40	50
10^{-7}	19	(A)	0	0	0	5	5	5
		(D)	0	0	0	5	5	5
10^{-1} Vaccinia	10	(A)	100					
		(D)	100					
10^{-2}	17	(A)	94	100				
		(D)	0	100				
10^{-3}	22	(A)	55	100				
		(D)	0	100				
10^{-4}	43	(A)	12	88	98	98	98	98
		(D)	0	58	98	100		
10^{-5}	25	(A)	20	52	56	56	56	56
		(D)	0	16	80	88	100	
10^{-6}	28	(A)	4	11	14	14	14	14
		(D)	0	4	7	11	18	18
10^{-7}	16	(A)	0	0	6	6	6	6
		(D)	0	0	19	19	19	19
10^{-8}	19	(A)	0	0	11	11	11	11
		(D)	0	0	5	11	11	16
10^{-2} Influenza NWS	14	(A)	50	100				
		(D)	0	86	100			
10^{-3}	29	(A)	14	90	100			
		(D)	0	48	93	100		
10^{-4}	27	(A)	26	93	100			
		(D)	0	41	100			
10^{-5}	19	(A)	5	21	47	47	47	47
		(D)	0	5	69	84	89	89
10^{-6}	15	(A)	0	13	20	33	33	33
		(D)	0	0	13	40	47	53
10^{-7}	20	(A)	0	0	0	0	0	0
		(D)	0	0	5	10	20	25
10^{-8}	14	(A)	0	0	0	0	0	0
		(D)	0	0	0	0	0	8

* (A) designates % of anomalous (microencephaly and/or axial flexion) embryos in each dilution while (D) indicates % dead within that group.

days after inoculation. All embryonic tissue pools were ground and then serially diluted with B.H.I. medium containing antibiotics in preparation for titration. With the exception of the distemper membrane pool, in which case 7-day embryonated eggs were employed, titrations of virus in each embryonic pool were conducted in 11-day eggs. The recovery of herpes, vaccinia, and distemper viruses was established by the detection of the characteristic lesions produced by each on the C-A membrane. The detection of NWS virus in the allantoic fluid was accomplished by the hemagglutinating method.

Results. Infection with herpes simplex, vaccinia and the NWS strain of influenza-A

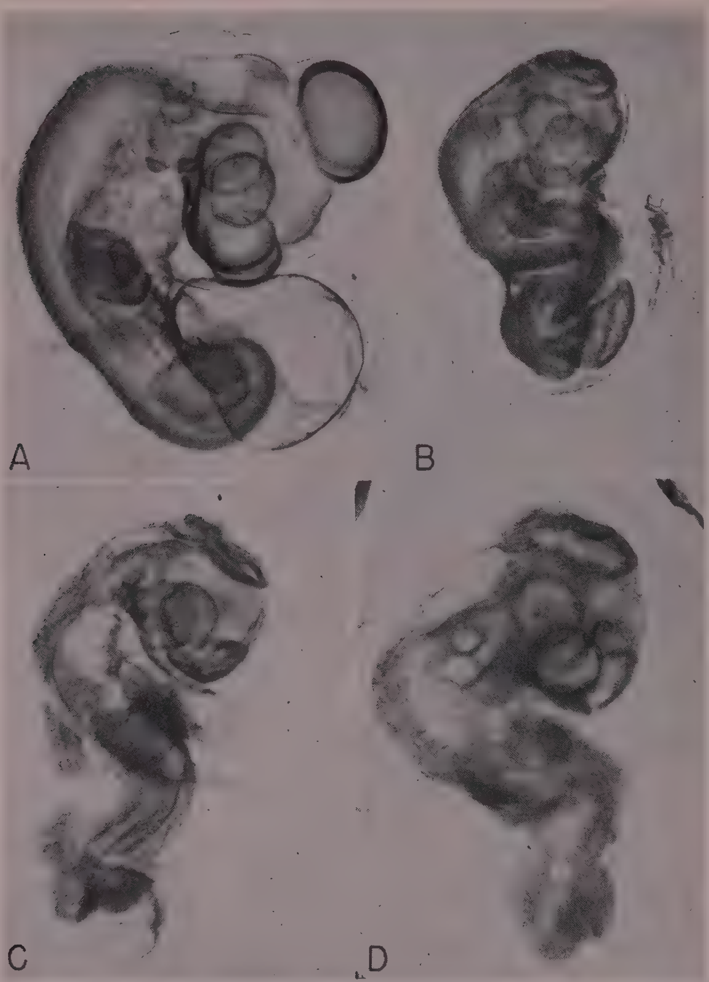

FIG. 2. Embryos after 4 days incubation. A. Uninfected control embryo showing well-developed brain and normal curvature of spinal axis. B. Embryo infected 48 hr previously with herpes simplex virus, showing flattened brain vesicles, axial flexion, and antero-posterior compression of eyes and auditory vesicle. C. Embryo infected 48 hr previously with vaccinia virus, showing micrencephaly, axial flexion, and optic and auditory damage. D. Embryo infected 48 hr previously with influenza-A (NWS) virus, showing swollen auditory vesicle in addition to micrencephaly and axial flexion.

viruses produced teratogenic effects which were quite similar to those previously described for the PR8 strain of influenza-A virus(2,3). In most cases, the first indication of infection was the appearance of a dorsal flexion of the body axis at the level of the wing buds or middle trunk region. The brain subsequently sustained extensive damage leading to more or less pronounced micrencephaly through the collapse of the major brain vesicles, *i.e.* the telencephalon and mesencephalon. The growth of the amnion was retarded in a majority of cases, although it appeared to have closed normally in others. In a few cases, the amniotic sac became turgid through internal fluid pressure. Thus, at the time of death infected embryos typically exhibited extreme axial flexion and advanced micrencephaly shown in Fig. 2. Table I shows that the incidence of anomalous devel-

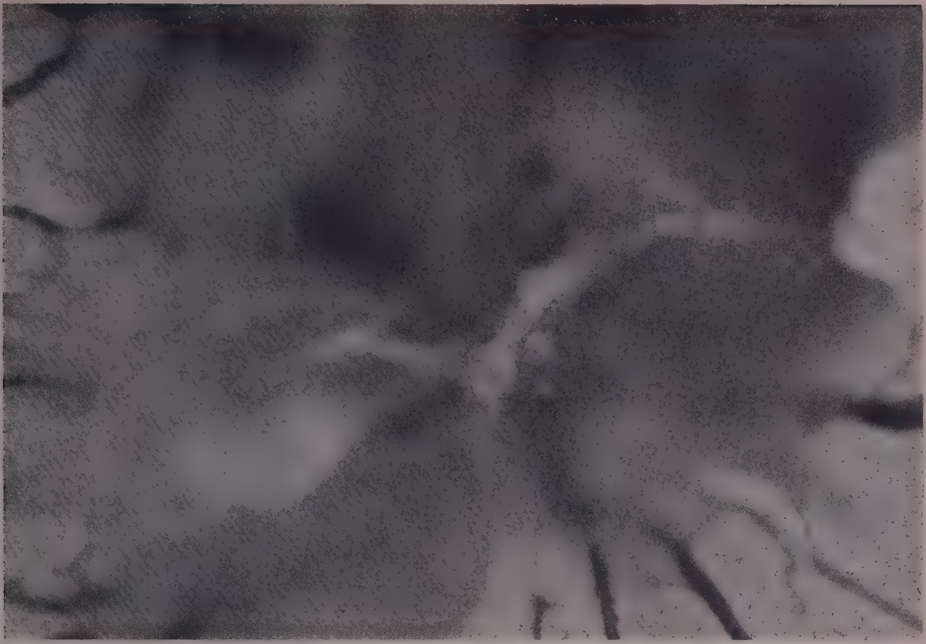

FIG. 3. Lesions on chorionic membrane directly overlying 4-day-old embryo inoculated 2 days previously with influenza-A (NWS) virus.

opment, time until appearance of anomalies, and time until death were functions of the virus dilutions employed. Once anomalies became apparent, subsequent growth of the embryo proceeded at a slow rate until death supervened. Infected embryos which failed to show anomalies by the sixth day appeared normal at death.

There were, however, minor differential effects which were characteristic of each virus. *Herpes simplex virus.* When herpes simplex virus was used as the inoculum, the major damage was similar to that produced by the other viruses, but the eyes tended to become compressed along their antero-posterior axis in a higher percentage of cases than was observed with the influenza-A or vaccinia viruses (Fig. 2B). The auditory vesicles of practically all herpes-infected embryos underwent severe damage and became very small white masses of tissue during the infection. *Vaccinia virus.* When embryos were infected with vaccinia virus, the eyes occasionally became flattened medio-laterally, and the auditory vesicles were in many instances either small and opaque or markedly compressed (Fig. 2C). The amnion sometimes appeared abnormally opaque along its free border with pox-like, raised lesions observable at the time of death. *Influenza-A (NWS strain) virus.* A striking effect, not noted when any other virus was employed, could be observed in embryos inoculated with NWS strain virus. About 2 days after injection, small, discrete, white lesions appeared on the extra-embryonic membranes in most cases, and frequently became contiguous and much enlarged by the time of death (Fig. 3). In addition, patches of whitish tissue could sometimes be seen along the borders or within the limb buds, in the branchial area, as well as near or in the somites and neural tube walls. The developing eyes frequently appeared flattened medio-laterally and were irregular in outline. The auditory vesicles were swollen in many cases, a condition not noted in embryos infected with the other viruses employed (Fig. 2D).

When tissue pools obtained from anomalous dead embryos infected with herpes, vaccinia, and NWS viruses were titrated it was

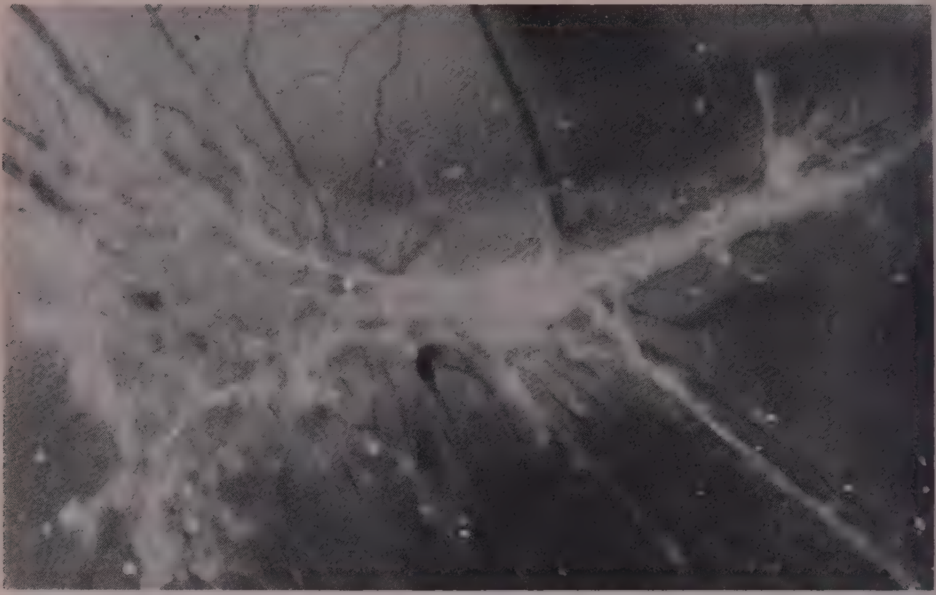

FIG. 4. Lesions on C-A membrane of 8-day-old embryo inoculated 6 days previously with distemper virus.

found that all 3 agents had undergone multiplication in the embryo during the period of "acquired" teratism to the extent that such pools could be diluted at least 10^6 and still yield detectable virus. Significantly, these 3 viruses, following heat inactivation, failed to evoke teratogenesis.

Unlike the other 3 viruses, distemper virus suspensions failed to produce in the embryo itself any gross morphological anomalies observable under the dissecting microscope. However, 4 or 5 days after injection of a 20% suspension of virus, small spot or thread-like lesions began to appear on the C-A membrane. During the next few days, these lesions grew in size and coalesced to form the large, ulcerative areas on the surface of the membrane (Fig. 4) which characterize distemper infection in older embryonated eggs (8). In a few cases, similar though smaller lesions could be detected on the amnion as well. It can be seen from Table II that almost all embryos possessed C-A lesions 7 days after inoculation with the 20% virus suspension, and that many had succumbed to the infection by that time. Most of those remaining had died by the tenth day, and all were dead 2 weeks after injection. Similarly, all embryos which developed C-A lesions following inoculation with 2% and 0.2% suspensions subsequently died of the infection. However, a number of embryos apparently

TABLE II. Effect of Distemper Virus Infection upon Early Chick Embryos.

Virus conc. (%)	No. of embryos	Effects observed*	Days following inoculation							
			3	4	5	6	7	8	9-13	14-18
20	43	(L)	0	2	37	60	98	100		
		(D)	0	0	0	2	21	74	98	100
2	21	(L)	0	10	19	29	67	81	81	81
		(D)	0	0	0	0	19	43	67	81
.2	8	(L)	0	0	0	13	38	38	38	38
		(D)	0	0	0	0	0	13	38	38

* (L) designates % with C-A lesions in each dilution group while (D) indicates % dead within that group.

TABLE III. Effect of Control Inocula upon Early Chick Embryos.

Inoculum	No. of embryos	% dead on days following inoculation 1	2	3	4	5	6-14
B. H. I. medium	40	3	5	8	8	8	8
Normal allantoic fluid (10^{-4})	43	0	0	5	5	5	5
Normal C-A membranes (20% susp.)	47	2	2	9	9	9	9
Total	130	1	2	7	7	7	7

escaped infection completely, with the percentage of survivors inversely proportional to the virus concentration employed. The C-A lesions which were produced as a result of distemper infection in 48-hour embryos were shown to contain active virus. Inoculation of such membrane pools into 7-day embryos produced the characteristic lesions of distemper (8). Significantly, heat inactivated distemper virus was not capable of producing C-A lesions.

As shown in Table III, about 7% of control embryos inoculated with B.H.I. medium or suspensions of uninfected allantoic fluid or C-A membranes subsequently died within 14 days. Indications of retarded embryonic development or operational injury (*i.e.*, damaged blastoderm) were almost always associated with these incidental deaths. Although such deaths were occasionally accompanied by aberrant embryonic development resembling virus disease, these anomalies were readily distinguishable from the latter. Similar incidental mortality occurred in about 7% of those embryos which were inoculated with virus suspensions, and such cases were therefore not included in the data presented in Tables I and II. Deaths occurring after 14 days were randomly distributed in control and virus groups alike. The majority of such deaths are probably due to incubation difficulties associated with collapse of the air sac, such collapse being a requisite of the operational procedure.

Discussion. Infection with herpes simplex, vaccinia, and the NWS and PR8 strains of influenza-A viruses evokes teratogenesis and death in the early chick embryo. The fact that these viruses all produce the same major syndrome, including micrencephaly and axial flexion, indicates that they may share the ability to attack the same morphogenetic centers during critical stages of embryonic development. However, minor differences in pathology were observed which suggest that the teratogenic effects of one virus may, in some instances, be differentiated from those of another. For example, embryos infected with the NWS strain of influenza-A characteristically developed lesions on the extra-embryonic membranes as well as in certain regions of the body itself while those inoculated with the PR8 strain never exhibited this effect(2,3). Similarly, compression of the eye, when it occurred, was generally medio-lateral in the case of vaccinia and the influenza viruses while it was usually antero-posterior in the case of herpes simplex. Moreover, other investigations showing that infection with Newcastle disease virus preferentially affects the lens, auditory vesicles, and other structures to a lesser degree(4-6) provide even more striking evidence that different viruses may attack different sites.

The phenomenon of "acquired" teratism appears to be dependent upon the presence of an active viral agent since herpes, vaccinia, and NWS viruses all failed, following heat inactivation, to produce anomalies in susceptible embryos. That these agents undergo multiplication in the embryo was borne out by titration studies which showed that the virus concentration rose at least 100-fold during the period in which teratogenesis occurred.

The ability of some viruses to produce anomalous development of the early chick embryo should not imply, however, that teratogenesis may result from any virus infection. Distemper virus, for example, evoked no gross teratogenic effects but produced a lethal infection characterized by ulcerative lesions on the C-A membrane. Similar lesions are known to result when the egg-adapted strain of distemper virus is inoculated onto the C-A membrane of older embryos(8), but death rarely occurs. The ability of the infection to kill younger but not older embryos may lie in the greater susceptibility of the metabolic processes of the early chick embryo to the pathogenic effects of the virus(9).

It is noteworthy that the virus strains which have been shown in this laboratory to produce anomalous development of early embryos following injection through the vitelline membrane were also capable of producing a lethal infection in older (9-10 day) embryos when introduced extra-embryonically. Newcastle disease virus also shares this property(5,6). On the other hand, distemper virus, which did not regularly produce death in older embryos, has not been shown to produce teratogenic effects in early embryos, under the experimental conditions so far employed. The results observed with distemper are quite similar to the data reported for mumps virus(2). The latter agent, although undergoing multiplication in the allantoic sac, did not kill 8-day-old embryos, nor did it prove teratogenic in 48-hour embryos, even though these younger embryos succumbed to the infection within 5 days. It is possible, however, that teratogenesis might also result from infection with these viruses following sufficient modification of such factors as the time selected for inoculation, or the volume and concentration of the inoculum.

Summary. Herpes simplex, vaccinia and influenza-A (NWS) viruses produced teratogenic and lethal effects in the early chick embryo. The primary teratogenic effects of all 3 viruses were micrencephaly and axial flexion, but minor characteristic differences could be detected in embryos infected with each of these agents. Distemper virus failed to produce any gross embryologic changes, although its injection led to the development of ulcerative lesions on the C-A membrane and death of the embryo.

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Free and Apparent Amino Acids in Deproteinized Plasma of Normal and Uremic (Nephrectomized) Dogs.* (22581)

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It would be of physiological and clinical importance to determine whether the disturbances of urea and protein metabolism, known to occur in uremia, are accompanied by abnormal levels of plasma amino acids. Although total amino nitrogen is not elevated consistently in the blood of uremic patients (1,2), it appears possible that individual amino acids may vary markedly. Levenson *et al.*(3) observed that plasma amino nitrogen was near normal in patients with severe battle wounds and renal failure although plasma urea concentrations were as much as 30 times normal. All plasma amino acids were not depressed or elevated at any time but the concentration of some individual

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amino acids rose during the first week. Schmidt *et al.*(4) reported that the concentration of arginine, phenylalanine and tyrosine in uremic plasma was more than double that in normal plasma although considered to be within normal limits. According to Stein and Moore(5), the concentration of glycine was elevated in cystinuria and of phenylalanine in phenylpyruvic oligophrenia although the levels of free amino acids in the plasma of patients suffering from a number of diseases were within the normal range.

Deficiency of even one "essential" amino acid in the blood of uremic individuals could lead to decreased protein synthesis and explain certain clinical features of the uremic syndrome (*e.g.*, poor wound healing, diminished antibody formation and decreased hemoglobin synthesis). On the other hand, excess of an amino acid might contribute to the toxicity of uremia. Plasma from normal dogs and from dogs rendered uremic by nephrectomy was employed in the studies reported here in order to minimize the variability characteristic of human uremic patients.

Choice of analytical method was an important consideration in the present study where the primary objective was to characterize uremic plasma in terms of its normal and any abnormal metabolites with particular emphasis upon free and combined amino acids. Although Redfield and Barron(6) have shown that amino acids may be determined satisfactorily in hydrolysates of a purified protein (bovine serum albumin) by paper chromatography this method, according to Evered(7), is subject to large errors with biological fluids. Stein and Moore(5) have reported that nearly all of the 28 ninhydrin-positive substances in filtrates from normal human plasma were determined by column chromatography using a sulfonated polystyrene resin (Dowex 50-X4) with a calculated yield (4.02 mg %) of α-amino nitrogen in close agreement with the value (4.1 mg %) which Hamilton and Van Slyke(8) found by direct ninhydrin-CO_2 analysis. That the concordance of these overall results may not reflect a similar degree of accuracy for individual amino acids is indicated by the ± 10% error in recovery of added amino acids and by the possibility that bound amino acids may have been partially hydrolyzed by ion-exchange catalysis(9,10). From their data obtained on acid hydrolysates of normal protein-free plasma Stein and Moore concluded that although major quantities of conjugated amino acids do not appear in plasma, "The possibility still remains that certain forms of conjugated amino acids of unknown nature might exist in plasma and be removed during the deproteinization procedure, either by being bound to the protein-picric acid precipitate or to the Dowex 2 column." Microbiological assay, rather than column chromatography, was selected for the authors' use because of its potentialities for the determination not only of free amino acids but also of conjugated amino acids and possibly other types of substances stimulatory or inhibitory to the growth of the assay organisms.

Experimental. Healthy mongrel dogs, fasted overnight, were exsanguinated from the carotid artery under sodium pentobarbital (25 mg/kg) anaesthesia. The blood was received in siliconed vessels each containing 20 mg of heparin per liter of blood. As soon as possible after exsanguination the heparinized blood was centrifuged, the plasma was pipetted and the residual red cell mass was discarded. The plasma was stored at —20°C, a temperature at which amino acids in plasma are stable(11). For preparation of uremic plasma healthy mongrel dogs were anaesthetized as described and bilateral nephrectomy was performed through a midline abdominal incision in one stage. The animals were returned to their cages and offered 20 ml per kilo of 15% glucose in water daily but no medication, or other fluid or food. Those animals which survived a minimum post-operative period of 72 hours and exhibited signs of uremic toxicity (*e.g.*, twitching vomiting, convulsions) were anaesthetized with the minimum dose of pentobarbital, exsanguinated and the blood processed as described. Amino acids were determined only in those plasma specimens with blood urea nitrogen of at least 200 mg %.

Deproteinization was effected by adding per ml of plasma 1.5 ml of approximately 4%

TABLE I. Concentration of Apparent Amino Acids in Deproteinized Plasma of Normal Dogs.*

	Tungstic acid filtrate		Trichloroacetic acid filtrate
Amino acid	Hier(17), Hier & Bergeim(11,18)	Authors§	Authors
Arginine	33†(23–48)‡	4.1 (1.5–9.9)	19 (16–22)
Aspartic acid		5.1 (1.8–9.2)	4.4
Glutamic "	<6	27 (3.0–65)	12
Glycine		14 (8.4–19)	25
Histidine	12 (9.3–16)	11 (7.2–15)	11
Isoleucine	13 (8.7–20)	14 (11–17)	15
Leucine	21 (14–31)	23 (16–30)	26
Lysine	24 (15–32)	17 (3.6–38)	40 (26–54)
Methionine	12 (8.0–16)	5.3 (1.9–13)	9.4 (7.1–12)
Phenylalanine	12 (7.0–18)	12 (9.9–14)	13
Proline		14 (10–19)	16
Serine		8.8 (2.8–14)	28
Threonine	26 (15–35)	29 (22–37)	31
Tryptophan	10 (7.5–13)		
Tyrosine	11 (4.5–20)		
Valine	22 (15–30)	25 (16–39)	32 (24–39)

* Expressed in μg/ml of plasma. † Avg value. ‡ Range of values. § Samples ranged from 2 pooled or 1 pooled plus 3 individual, to 3 pooled plus 5 individual plasma samples from 13 normal dogs.

sodium tungstate in 0.35 N H_2SO_4 or 1.0 ml of 10% trichloroacetic acid solution. Each mixture was allowed to stand for 30 minutes with occasional shaking, the suspension was filtered on a Buchner funnel, the residue was washed twice with a minimum volume of distilled water and the washings were added to the filtrate. The combined washings and filtrate was extracted with ether to remove excess trichloroacetic acid(12).

Some of the solutions were assayed for amino acids without further treatment. Others were evaporated to dryness under reduced pressure to residual material which was taken up in warm distilled water and, after filtration, was diluted with distilled water to a volume estimated to contain amino acids in a concentration most suitable for the microbiological assays. The assay procedures have been described(13,14).

Results. A factor of critical importance to the present study concerns the capacity or incapacity of deproteinizing agents to precipitate or adsorb proteins, peptides and amino acids from plasma. Hamilton and Van Slyke (8) found that picric and tungstic acids—but not zinc, cadmium or ferric hydroxides—yielded filtrates without loss of amino acids while Christensen and Lynch(15) reported that tungstic acid, picric acid and trichloroacetic acid gave filtrates containing increasing amounts of conjugated amino acids in the order named. On the basis of the latter results Henderson *et al.*(16) adopted tungstic acid as the deproteinizing agent most suitable for the determination of amino acids in plasma by microbiological assay. Stein and Moore (5) concluded that it was more satisfactory to remove proteins with picric acid than by equilibrium dialysis or ultrafiltration.

In the authors' experiments it was found (Tables I and II) that the values for apparent free amino acids were higher for glutamic acid, lower for arginine, glycine, lysine, and serine and about the same for the other amino acids in plasma filtrates deproteinized with tungstic acid compared to trichloroacetic acid. These differences cannot be explained at present.

The concentration of apparent free histidine, isoleucine, leucine, phenylalanine, threonine and valine (Table I) found in tungstic acid filtrates of normal dog plasma agrees (average difference about 8%) with that obtained by Hier(17) and Hier and Bergeim (11,18) under comparable conditions. That these values may represent approximately the concentration of the free amino acids is indicated by the low (average, 3.2%; range, 1.6-7.5%) average mean deviations from the

TABLE II. Concentration of Apparent Amino Acids in Deproteinized Plasma of Uremic (Nephrectomized) Dogs and Its Relationship to That Found for Normal Dogs.

	Conc. in plasma*		Ratio of conc. (uremic/normal)†	
Amino acid	Tungstic acid‡	Trichloroacetic acid‡	Tungstic acid	Trichloroacetic acid
Arginine	8.8§(5.7–15)‖	38	2.5§(1.2–3.6)‖ (.16–3.0)¶	2.0 (1.7–2.4) (1.0–2.0)
Aspartic acid	7.8(3.3–19)	7.0	2.7 (1.0–4.5) (1.3–3.0)	1.6 (2.0)
Glutamic "	16 (6.7–47)		.35(.34–.36) (2.8–3.4)	
Glycine	31 (16–50)	40	2.9 (2.2–3.8) (.36–1.3)	1.6 (.40)
Histidine	16 (15–18)	12	1.0 (1.0)	1.1 (.40)
Isoleucine	17 (16–18)	15	1.1 (.65)	1.0 (.55)
Leucine	31 (26–36)	28	1.0 (.60)	1.1 (.50)
Lysine	13 (5.0–28)	16	.99(.71–1.4) (.26–3.0)	.62 (.55)
Methionine	4.4(1.9–12)	5.7	.63(.45–.78) (.24–2.1)	.81 (1.0)
Phenylalanine	16 (14–18)	14	1.2 (.52)	1.0 (.50)
Proline	14 (11–17)	16	1.0 (.89–1.1) (.88–1.3)	1.0 (1.0)
Serine	10 (6.0–18)	43	1.1 (.91–1.3) (.65–.87)	1.5 (.74)
Threonine	11 (9.7–13)	7.9	.22 (.55)	.26 (.58, 1.9)**
Valine	26 (21–32)	19	.94 (.52)	.79 (1.0)

* Expressed in μg/ml of plasma.

† Only at approximately equal concentrations of plasma.

‡ Deproteinizing agent.

§ Avg value. Samples ranged from 2 pooled or 1 pooled plus 5 individual, to 3 pooled plus 6 individual plasma samples from 14 nephrectomized dogs.

‖ Range of values.

¶ Range of plasma concentrations over which comparison was made.

** Relative concentration of normal and uremic plasma samples, respectively. No data at equal concentrations available.

means observed at the different levels of samples. Similarly, the comparable average mean deviations for proline (not determined by Hier or Hier and Bergeim) and glutamic acid were 2.0 and 3.8%, respectively. The relatively low value (<6 γ per ml) for glutamic acid obtained by Hier and Bergeim(18) appears to be explained by conversion of glutamine to microbiologically inactive pyrrolidone carboxylic acid during autoclaving of the plasma filtrate(19). The authors' relatively high value (average, 27 γ per ml, range, 3.0-65 γ per ml) may be due to partial hydrolysis of glutamine (to glutamic acid) during evaporation to dryness of the acidic tungstic acid filtrate prior to autoclaving in the assay. According to Archibald(20) deproteinized plasma from fasting dogs contains 70-130 γ per ml of glutamine. Similarly, the predominance of asparagine rather than aspartic acid in plasma(5) probably accounts for the inability of Hier and Bergeim to find detectable amounts of aspartic acid while the present authors found appreciable quantities (average, 5.1 γ per ml, range, 1.8-9.2 γ per ml).

The authors believe that low mean deviations from the mean of assay values at different levels of test sample signify that significant quantities of microbiologically active compounds other than the test substance were

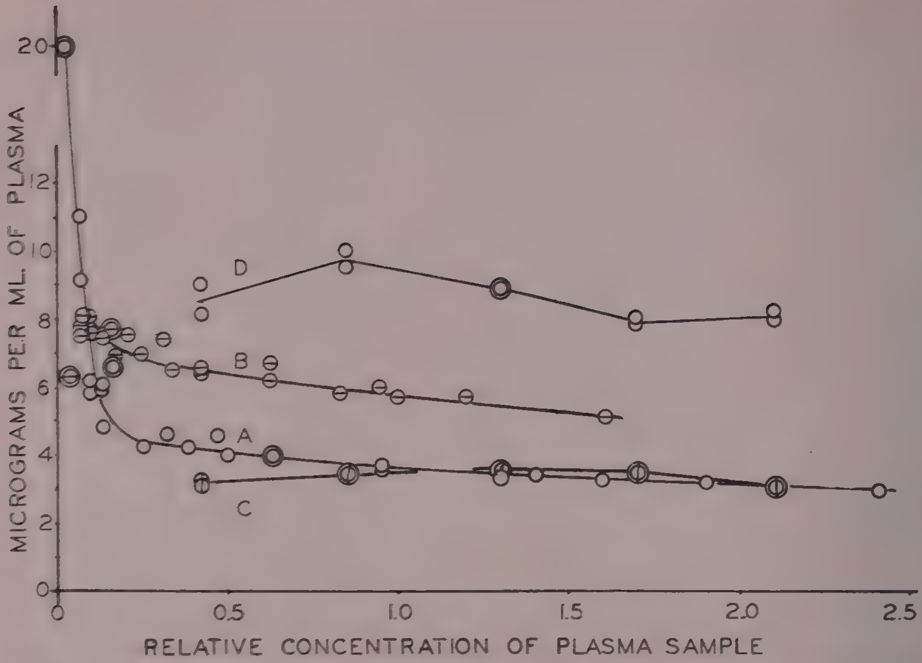

FIG. 1. Concentration of apparent arginine in deproteinized plasma of normal and uremic (nephrectomized) dogs. A—Normal plasma, unhydrolyzed; pooled sample from 3 dogs. B—Uremic plasma, unhydrolyzed: sample from 1 dog. C—Normal plasma, partially hydrolyzed; pooled sample from 3 dogs. D—Uremic plasma, partially hydrolyzed; pooled sample from 5 dogs.

not present. This criterion is believed to be valid, however, only for assays in which the plasma filtrate is assayed over at least 5-fold concentration of which the highest is equal to or greater than that of the original plasma. The discordant values (relatively high average mean deviations from the means) found at the different levels of samples for the other amino acids resulted, presumably, because of the presence in deproteinized plasma of conjugated amino acids and possibly other types of substances stimulatory or inhibitory to the growth of the assay organisms. The markedly different values found for apparent free arginine (Fig. 1) at varying concentrations of plasma sample provide a striking illustration of the point that assay data of this character have only relative significance and that only at the same concentrations of plasma sample.

The ratios (Table II) of the concentrations of apparent amino acids in the plasma filtrates from uremic (nephrectomized) compared to normal dogs were calculated from values obtained *only* at equal concentrations of normal and uremic plasmas. It is of interest, however, that the ratios (Tables I and II) calculated for all of the plasma samples are of the same order of magnitude. It is evident that the uremic to normal plasma ratios are significantly greater than 1 for arginine, aspartic acid and glycine and are significantly less than 1 for glutamic acid and threonine. Although the ratios for glutamic acid and aspartic acid may be fortuitous for reasons mentioned earlier in this paper, those for the other amino acids listed are believed to be valid. Supporting this view is the observation(21) that the activity of hippuric acid towards *L. mesenteroides* P-60 is not markedly different than that of glycine and the present authors' (unpublished) finding that these results cannot be attributed to creatine, creatinine or urea metabolites known to be present in relatively high concentration in uremic blood. Whether or not these different ratios of amino acids plus amino acid-like components are characteristic of normal compared to uremic human plasma filtrates is now under investigation in the authors' laboratory. Whether or not such altered concentrations of amino acids and any other compounds in plasma filtrates which affect growth stimuli of microorganisms bear any relation to the physiological or clinical aspects of uremia remains to be determined.

The increased concentration of plasma arginine in uremic dogs could be explained by the accumulation of urea with concomitant slowing of the formation of urea and ornithine from arginine. The expectation that there would be an accompanying low concentration of ornithine in plasma is now being investigated. Although there is no obvious explanation for the accumulation of apparent glycine in uremic plasma it is known that excessive glycine produces toxic effects. The low concentration of threonine and, possibly, methionine in uremic dog plasma may be a factor in decreasing protein synthesis.

Summary. 1. Microbiological assay procedures were found to be applicable to the determination of free amino acids in deproteinized plasma but only in the absence of interfering substances. The apparent (relative) values obtained in the presence of interfering substances were shown to be meaningful but only when determined over a wide range of concentration of plasma filtrates. 2. It was concluded that the concentration of some free amino acids (histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine and valine) was not significantly different but that of some apparent amino acids was higher (arginine and glycine) and others (methionine and threonine) lower in the deproteinized plasma of uremic (nephrectomized) compared to normal dogs.

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Mortality and Skin Transplantability in X-Irradiated Mice Receiving Isologous, Homologous or Heterologous Bone Marrow.*† (22582)

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The protective effect of intravenous administration of bone marrow or spleen cell suspensions after an otherwise lethal dose of x-irradiation is well established. Such protection is usually associated with rapid hematopoietic recovery. Early observations usually involved short term protection using cell suspensions from the same strain of animals as the irradiated recipient. Later it was reported that some degree of irradiation protection could be obtained with homologous‡ and even heterologous marrow(1-4). Barnes and Loutit(5-6) failed to obtain protection with heterologous spleen or marrow, and have called attention to the importance of evaluating protection with homologous marrow beyond the usual 30 day end-point. It is well established that most tissues of one inbred strain of mice may ordinarily be transplanted with success to all other members of the same inbred strain, but not to members of another strain, unless that strain contains all of the histocompatibility genes of the donor strain (7). Thus, tissue may be transplanted successfully from either inbred parent strain to the F_1 hybrid, but not vice versa. Main and Prehn have reported that in mice protected with marrow from an F_1 hybrid of the irradiated strain, it was subsequently possible to successfully homograft skin from the other (foreign) parental strain. Such skin grafts sloughed in mice protected with isologous marrow(8). The present study was undertaken to determine the relative degree and duration of protection afforded by isologous, homologous, and heterologous sources of bone marrow in irradiated mice, and to extend the observations of Main and Prehn on skin homografting.

Materials and methods. Bone marrow preparation: Mice of the following inbred strains were employed: CBA, Strong A, Cb, C_{57}, C_3H, Db and their F_1 hybrids A x Db and Cb x CBA. The Cb and Db strains are the Balb/c and Dba strains without the mammary tumor inciter. Mice received total body x-irradiation in groups of 6 under the following conditions: 200 KVP, 15 Ma, 40 cm target distance, 0.5 mm Cu and 1.0 mm Al filters, HVL = 1.3 mm Cu, 88-101 r/minute dose rate. Calibration with a Victoreen dosimeter placed in the mouse chamber was carried out each day the machine was used.§ In preparing bone marrow suspensions from mice, 6 bones from each young adult donor mouse (femora, humeri, tibias), were scraped free of muscle, the marrow cavity was broken open at both ends, and a tight fitting hypodermic needle on a 1 ml syringe was inserted into one end. The bone was then submerged in a tube of saline solution (.8% NaCl, .02% KCl) and the marrow flushed from the bone by drawing saline in and out of the syringe. Saline was used at the rate of .5 ml per donor mouse. The washings were pooled and the small amount of coarse particles allowed to settle out by gravity. The remaining super-

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‡ The terms autologous, isologous, homologous, and heterologous are used as defined by Snell(7): autologous—from same individual; isologous—from another individual of same inbred strain (or identical twin); homologous—from individual of different genotype but of same species; heterologous—from different species.

§ X-ray facilities and calibration were generously provided by the Radiation Physics Department of the M. D. Anderson Hospital and Tumor Institute.

natant fluid was essentially a single-cell suspension. Cell counts made by the method for white cells in peripheral blood gave counts on the order of 50,000 cells per cu mm. A sample of each cell suspension was spun down, smeared and stained for microscopic examination. The suspension was administered (.5 ml per mouse) via the tail vein, under sodium amytal anesthesia, within 1 to 3 hours after irradiation. Aseptic technic was used throughout. *Rat marrow* was similarly collected from 5 Sprague-Dawley rats and suspended in 16 cc of saline. Each irradiated mouse received .5 ml. *Guinea pig marrow* was similarly collected from each of 5 pigs, 8 days old, using 13 ml of saline. The suspension was spun down at 200 x G for 10 minutes, the supernatant fluid discarded, the cells resuspended in saline, and the process repeated. The cell count on the material injected was 70,350 per cu mm. Volume injected was .5 ml. *Rabbit marrow* (4 ml) was collected by aspirating the marrow cavities of 6 bones from a 5.8 kg albino male. This was dispersed in 10 ml saline, and fat and debris removed by centrifugation. Saline was added to bring the volume of the cell suspension up to 13 ml. The volume injected per mouse was .5 ml with a cell count of 13,550 per cu mm. One batch of isologous mouse marrow was fractionated into a washed cell fraction and a pooled supernatant plus washings fraction. This batch was prepared initially with 4 ml of saline for 6 donor mice. The cells were spun down at 200 x G, the supernatant pipetted off and the cells resuspended in 1.5 ml saline. This was repeated a total of 3 times. The fourth time the cells were resuspended in 7 ml saline and .5 ml injected per irradiated mouse. The 4 supernatants were pooled and freed of cellular contamination by centrifuging at 1000 x G for 20 minutes, the sediment discarded, and the supernatant spun again at 7000 x G for 5 minutes. This final supernatant fluid was injected (.5 ml per irradiated mouse). Another batch of isologous mouse marrow was prepared in the usual way except that distilled water was used instead of saline to flush the marrow from the bones, in order to lyse the cells (no intact cells could be found in the smear). *Skin grafts*: Irradiated mice that were well-protected by isologous or F_1 hybrid, or foreign strain mouse marrow were skin-grafted in from 33 to 161 days post-irradiation. Under sodium amytal anesthesia the hair was removed from the dorsum of the mouse with electric clippers. A piece of skin approximately 2 cm square was removed and replaced with a piece of skin of the same size from the same strain, or a foreign strain, or an F_1 hybrid of the irradiated strain. Interrupted silk sutures were used. No dressing or bandage was applied. The mice were individually penned until the graft had sloughed or was definitely healed. When skin from the same strain was grafted in an unirradiated control the graft was an autograft; the piece of skin removed was turned 180° and sutured back into the bed from which it came. Cortisone treatment consisted of 5 mg per day of cortisone acetate intraperitoneally in 2 divided doses, morning and evening, for 11 days. Bone marrow suspension from the F_1 hybrid was injected at 1, 3 and 13 days following the last injection of cortisone.

Results. The 21-day LD_{50} was approximately 600 r. At 770 r mortality was 100% for 136 mice, the longest survival being 12 days. At this level of irradiation, which was selected as a standard against which to measure protection, the peripheral leukocyte count fell to a few hundred cells per cu mm by the third day and remained low until death. Platelets became difficult to find in the blood smears by the fifth day. Body weight declined progressively until death. Mice dying before the seventh day showed no hemorrhage, but after the eighth day hemorrhage of the gastrointestinal tract and brain was a very common autopsy finding. *Mice receiving isologous bone marrow* after irradiation showed the same severe depression of circulating leukocytes by the third day, followed by a rapid return to the normal range as early as the seventh day. Mortality by the 12th day, when all of the controls were dead, was only 13%, and there was very little further mortality as late as 100 days post-irradiation (Table I). Protective activity was exhibited by the washed cell fraction but not by the cell free supernatant fraction or by the lysed cell

TABLE I. Cumulative Mortality in Mice Receiving 770r followed by Isologous, F$_1$ Hybrid, Foreign Strain, and Heterologous Bone Marrow.

Source of marrow	Irradiated mouse strain	No. of mice	Marrow donor, strain or species	% mortality after indicated No. of days 12	21	30	60	100
Same strain of mice	A	12	A	0	0	0	0	0
	Cb	11	Cb	55	55	55	55	64
	CBA	12	CBA	0	0	0	0	0
	CBA	12	CBA	0	0	0	17	19
	All combined	47		13	13	13	17	19
F$_1$ hybrid mouse	A	10	A × Db	30	50	50	80	90[†]
	A	12	A × Db	0	0	8	8	16[‡]
	A	10	A × Db	40	40	60	80	90
	CBA	12	Cb × CBA	0	0	0	0	0[§]
	All combined	44		16	20	27	39	45
Foreign strain of mice	A	12	Db	0	17	42	100	100
	CBA	12	C$_3$H	8	8	8	33	33[‖]
	CBA	12	A	8	8	25	75	83
	Cb × CBA	11	A	18	27	36	45	[*]
	C57	7	A	0	0	0	100	100
	All combined	54		8	15	28	69	
Foreign species	Cb	31	Rat	45	71	77	94	97
	CBA	24	Rabbit	83	96	96	96	96
	A	24	Guinea pig	83	100	100	100	100

[*] Has not yet reached 100 days post-irradiation.
[†] Of 3 mice skin grafted at 43 days, 1 died at 57 days, 1 at 90 days.
[‡] Of 8 mice " " " 74 to 77 days, all lived beyond 100 days.
[§] All 12 mice skin grafted at 30 to 56 days.
Of 6 mice skin grafted at 71 to 72 days, all lived beyond 100 days.

suspension. *Marrow from an F$_1$ hybrid or foreign strain* of mice likewise resulted in rapid recovery of circulating leukocytes, and protected against mortality just as effectively as isologous marrow until the 21st day. Between the 21st and 60th day however, mortality increased steadily and significantly in the foreign marrow group, in spite of circulating levels of red cells, white cells and platelets within the normal range (with the exception of one mouse that showed a terminal erythropenia). Late death in these mice was usually preceded by progressive loss of body weight. The only apparent gross pathology at autopsy was pulmonary congestion and consolidation. *Marrow from F$_1$ hybrids* was, on the average, intermediate in protective activity between isologous and foreign strain marrow. Variation between groups receiving F$_1$ marrow, however, was considerable. Two such groups showed excellent protection as late as 100 days post-irradiation. In the peripheral blood of the mouse, lymphocytes normally outnumber polymorphonuclear leukocytes in the ratio of about 2 to 1. In the mice surviving 770 r as a result of receiving either isologous, F$_1$ hybrid, or foreign strain mouse marrow, this ratio was reversed. *Irradiated mice receiving guinea pig or rabbit marrow* showed 83% mortality by the 12th day and were all dead by the 21st day except one mouse. This mouse received rabbit marrow and is still alive at 234 days. It survived in spite of a very slow recovery of circulating white cells (950 cells/cu mm at 16 days, 2800 at 22 days; 10,500 at 29 days). At no time were granulocytes characteristic of the rabbit seen in the peripheral blood of this mouse. (Mouse and rat neutrophils are essentially agranular, while rabbit and guinea pig neutrophils have characteristic granules which distinguish them morphologically from those of the mouse.) *Irradiated mice receiving rat marrow,* although not as well protected as those receiving mouse marrow, showed significant protection on the 12th day. By the 21st day mortality in the rat marrow group had increased significantly, and by 60 days only 2 mice were still alive. These results with rat marrow are very similar to those reported by Cole *et al.*(3). *Skin grafts.* Of 34 autografts in unirradiated control mice, 3 sloughed, presumably because of

TABLE II. Results of Skin Grafting.

					Takes		Sloughs	
Host strain	X-ray dose (r)	Marrow donor	Skin donor	Day post-r when grafted	No.	Days duration to date of writing	No.	Days duration
A	None	None	A	—	7	142–162	2	13–14
CBA	"	"	CBA	—	24	57–140	1	<27
"	"	"	C_3H	—	0		4	17–19
"	"	"	Cb	—	0		9	<21
A	880	A	A	33	2	253	0	
"	770	"	A × Db	161	0		5	25–33
"	"	"	Db	161	0		6	22–40
"	"	A × Db	A × Db	43–77	1	Dead at 48	2	<21
					4	143–260		
"	"	"	Db	74–77	2	143	2	<24
CBA	660–770	Cb × CBA	Cb	35–56	15	57–79	3	13–16
"	770	C_3H	CBA	71	2	140	0	
"	"	"	C_3H	71–72	4	139–140	0	

inadequacies of technic (Table II). Of 13 homografts in unirradiated control mice, all sloughed. Of 22 irradiated mice protected with marrow from an F_1 hybrid and homografted with skin from the other parent strain, only 5 sloughed. The other 17 grafts, after an initial shedding of hair, regrew hair characteristic of the donor skin and are still in good condition 57 to 143 days after grafting.

Of 54 mice protected with foreign strain marrow, only 6 CBA mice that received C_3H marrow remained in good enough physical condition for long enough to warrant skin grafting. Of these, 2 were grafted with isologous skin, and 4 with homologous skin from the same strain that donated the bone marrow. All 6 grafts took and have persisted for 139 to 140 days. It may be significant that the CBA and C_3H strains are closely related in origin, and that tumors arising in one strain sometimes transplant to the other.

In irradiated mice protected with isologous marrow and homografted with skin from a foreign strain or an F_1 hybrid, all 11 grafts sloughed, indicating a specificity of the tolerance of skin homografts in irradiated marrow-protected mice. Only if the marrow used for protection came from the same foreign strain as the skin donor, or from an F_1 hybrid of the skin donor strain, was tolerance of the foreign skin exhibited.

Of the irradiated mice included in the mortality data, 29 mice that appeared well-protected were skin grafted during the 100-day period for which mortality data is presented. Skin grafting did not significantly alter the mortality data. Only 2 of the skin grafted mice died during the 100-day period.

Forty-five mice were started on cortisone treatment followed by F_1 hybrid bone marrow. Twenty-three mice survived the treatment. From 17 to 61 days after the last injection of cortisone, 12 of these mice were grafted with skin from the F_1 hybrid and 9 with skin from the other parent strain. In all 21 cases the grafts sloughed.

Discussion. The closer the genetic relationship, the greater is the protective activity of the marrow. The importance of evaluating the longevity of protection beyond the 21- or 30-day endpoint is apparent from the results obtained with rat marrow and foreign strain mouse marrow. Successful homografting of skin after protection with marrow from an F_1 hybrid of the irradiated and skin donor strains, but not after protection with isologous marrow, agrees with the results of Main and Prehn(8). That F_1 marrow is not unique in this respect is indicated by the successful homografting of skin, in the 4 cases in which this was tried, after protection with marrow from the same foreign strain as the skin donor.

There is increasing evidence supporting the concept that the tissue homograft reaction is an immunological response to foreign tissue antigens(7,9,10,11). Homologous mouse bone marrow in addition to permitting survival by virtue of hematopoietic recovery, appears also to have induced immunologic tolerance of tissue antigens from the marrow donor source. Main and Prehn(8) have called attention to the possibility that the mechanism of the latter effect may be similar to the induced immunological tolerance of foreign strain skin homografts in mice that have been injected as fetuses with tissue suspensions from the skin donor strain(12). The initial failure to induce tolerance to skin homografts by cortisone treatment and F_1 hybrid marrow does not eliminate this possibility. Pertinent to the question of whether survival of foreign strain marrow and skin after irradiation results from an induced immunological tolerance, in the sense of Billingham, Brent and Medawar, is the following: After protection with homologous or heterologous marrow against an otherwise lethal dose of x-irradiation, does the host's antibody producing tissue survive in a functional sense, or is it completely replaced by the comparable system of the marrow donor? Only in the first case must one postulate an altered immunological specificity of the surviving host system.

It has recently been shown that in irradiated mice and rats protected with homologous marrow, and in irradiated mice protected with rat marrow, the introduced cells survive and repopulate the irradiated host's otherwise depleted hematopoietic tissues(13-16). The failure of heterologous marrow to afford as great protection as any kind of mouse marrow in the first 2 or 3 weeks after irradiation may be related to an inability of heterologous marrow in a foreign host to perform all of the vital functions performed by homologous or isologous marrow. On the other hand, while rat marrow persists and proliferates in the irradiated mouse, it is possible that guinea pig and rabbit marrow, because of a greater genetic dissimilarity, does not. The failure to find granular leukocytes characteristic of the rabbit in the blood smears of the one rabbit-marrow-injected mouse that did survive indefinitely suggests this possibility.

The late mortality in irradiated mice receiving foreign strain mouse marrow is of interest, since it occurs in spite of 21-day protection equal to that with isologous marrow. Does this represent a delayed immunological response of the host to the tissue of the foreign strain as Barnes and Loutit(6) and Cole *et al.*(15) have suggested to explain late mortality in mice protected with homologous or heterologous bone marrow? The essentially normal blood counts before late death of mice receiving foreign strain mouse marrow, and the long duration of tolerance to skin homografts do not accord with this hypothesis. Perhaps more likely is the opposite possibility. The evidence for survival of marrow cells from a foreign strain or foreign species, and of skin from a foreign strain, indicates an induced immunological tolerance for tissues from the foreign marrow donor, on the part of any surviving antibody producing tissue of the irradiated host. But is there adequate reason to expect that the reverse has been accomplished? If the introduced foreign strain mouse or rat marrow survives and proliferates and produces cells capable of antibody production, this antibody producing system, by virtue of its foreign origin, should be immunologically active against all of the tissues of the irradiated host. Only the antibody producing systems of the same inbred strain or of an F_1 hybrid should not be immunologically active against the irradiated strain.

Summary. The degree and duration of protection afforded by isologous, homologous and heterologous sources of bone marrow administered after lethal doses of x-irradiation in mice was directly proportional to the closeness of genetic relationship of the marrow donor to the irradiated mice. Uninjected controls were all dead by the 12th day. Mouse marrow from the same strain or from an F_1 hybrid of the irradiated strain, or from a foreign strain, all gave excellent and essentially equal protection as late as 21 days post-irradiation. While mice receiving marrow from the same strain remained well protected beyond 100 days, those receiving marrow from a foreign strain showed considerable late mortality between the 21st and 60th days. Mortality in mice receiving F_1 hybrid marrow was intermediate at 60 days. Guinea pig and rabbit

marrow elicited a very low degree and duration of protection. Rat marrow permitted 55% of the irradiated mice to survive beyond the longest survival of the untreated controls. The duration of protection however was short, only 6% at 60 days. Irradiated mice protected with marrow from an F_1 hybrid of the irradiated strain showed a high degree and long duration of tolerance of skin homografts from the F_1 hybrid or from the other parent strain. Irradiated mice protected with foreign strain mouse marrow showed a high degree and long duration of tolerance for skin homografts from the same foreign strain. Irradiated mice protected with marrow from the same strain did not tolerate skin homografts from a foreign strain or from an F_1 hybrid.

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I^{131} and P^{32} Accumulation in Anuran Thyroid Gland in Normal and Accelerated Metamorphosis.* (22583)

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The application of radioactive tracers to studies of amphibian development, though infrequent, reveals that the tadpole thyroid can concentrate radioactive iodine and that this process may be influenced by thyroid stimulating hormone (TSH). Employing autoradiographic and histologic methods, Gorbman and Evans(1) demonstrated that radioiodine accumulation in the developing thyroid of Hyla larvae began soon after folliculation appeared in the gland. Autoradiographs became progressively darker as the thyroid increased in size and the colloid increased in amount. Dent and Hunt(2) found marked localization of I^{131} in autoradiograms of the thyroid from Hyla, Bufo, and Rana larvae, with no diminution in thyroidal radioactivity observed throughout metamorphic and post-metamorphic processes. Money, Lucas, and Rawson (3) measured the turnover of I^{131} in normal, thiouracil, and TSH treated *Rana pipiens* tadpoles. Radioactivity of the area of tissue bearing the thyroid ("block dissection") approximated 10-20% of the administered radioactivity. No substantial difference was found between metamorphosing and non-metamorphosing tadpoles. Surprisingly, the administration of TSH usually decreased rather than increased thyroidal I^{131} collection. In the main, the results of these experiments with radioactive tracers do not conform to those

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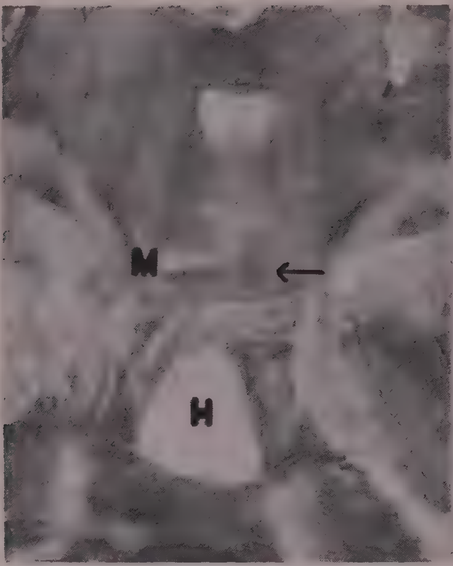

FIG. 1. Photograph (× 12) of freshly dissected hypobranchial region of a young tadpole. Transverse hyoglossus muscle (M) has been removed to expose one thyroid lobe (arrow). Ventral aortic roots and heart (H) have been left *in situ*. (Anatomic relations of developing anuran thyroid have been described(6).)

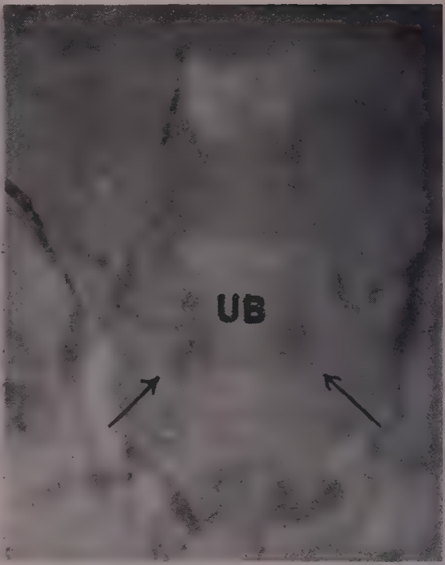

FIG. 2. Photograph (× 18) of hyoid area, with glossal muscles and heart removed. Thyroid lobes are shown (arrows), one on each side of urobranchial cartilage (UB). Vascularization and folliculation of the glands are recorded.

obtained from earlier morphologic and physiologic studies. The sequence of growth and histologic changes which characterize the thyroid throughout anuran metamorphosis are known to depend importantly on adequate thyrotrophic hormone function in the developing tadpole and can be precociously induced with exogenous thyrotrophin(4-9). The present study attempts to provide a more critical evaluation of functional changes in the larval thyroid by direct measurement of radioactivity in the gland isolated from Rana tadpoles after the injection of P^{32} and I^{131}. The avidity of the thyroid for these radioactive isotopes will be compared in normal metamorphosis and for accelerated development induced with exogenous TSH.

Materials and methods. Rana clamitans larvae were obtained from the field in various stages of hindlimb development during the winter, fall, and spring seasons. (This species is partially neotenic; metamorphosis of the tadpole is deferred for approximately one year.) The tadpoles were maintained at laboratory temperature (20-23°C) in aerated aquaria containing spring water and ample amounts of sphagnum moss as the only source of food. During the injection period, larvae were kept in shallow enamel pans and food was withheld. All injections were made into the body cavity by a method routinely used for bioassay(10). Tadpoles were given 0.5-1.0 μc of I^{131} or P^{32} in 0.1 ml of carrier free solution[†] 3 to 4 hours prior to sacrifice. Thyrotrophin,[‡] (Parke, Davis preparation and the U.S.P. Reference Standard), was administered in .05-.1 ml of acid saline. Tadpoles received the last injection of TSH 23-25 hours before measurement of radioactivity in the thyroid. At sacrifice (decapitation), the hypobranchial region and adjacent pericardial sac were exposed (Fig. 1) by removal of in-

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[†] Radioactive isotopes were obtained from Oak Ridge National Laboratory, Tenn.

[‡] We express our thanks to Dr. W. Donaldson of Parke, Davis and Co., Detroit, Mich., and to Dr. Adley B. Nichols of the U. S. Pharmacopeia for supplies of the hormone.

TABLE I. Comparison of Thyroidal I^{131} and P^{32} Uptake in Normal Metamorphosis.

Exp. No.	Stage of development* H.L.	B.L.	No. of animals	Mean thyroid I^{131} uptake		Mean thyroid P^{32} uptake	
				% of inj. dose (10^2)			
1	3.3 ± .5	22.5 ± 1.2	19	11.7 ± 1.8	P <.001‡		
	13.9 ± 1.9	29.5 ± 1.0	18	49.1 ± 6.3			
	1.8 ± .3	18.9 ± 1.0	8	11.6 ± 3.3	P <.001		
	25.0 ± 2.4†	24.0 ± 1.2	4	242.0 ± 54.0			
	2.3 ± .5	22.0 ± 1.0	16			.49 ± .09	P n.s.
	15.1 ± 2.3	30.4 ± 1.4	14			.74 ± .15	
2	2.8 ± 1.0	21.2 ± 1.9	12	7.0 ± 1.0	P <.001		
	13.5 ± 2.5	25.0 ± 1.5	8	22.8 ± 3.6	P <.001		
	34.0 ± 1.0†	24.5 ± 1.0	2	89.7 ± 5.0			
	1.4 ± .3	19.0 ± 1.0	8			.25 ± .07	P <.001
	25.9 ± 2.0†	25.5 ± 1.2	8			.97 ± .08	

* H.L. and B.L. refer to mean hindlimb and body lengths in mm ± avg dev. In other columns, values represent mean ± stand. error of mean.

† Animals in advanced stages of metamorphosis (imminent or actual forelimb emergence).

‡ Statistical significance calculated from "T" test tables. "P" values >.05 are not considered significant (n.s.).

tegument and subjacent musculature. The "os thyroideum" (urobranchial cartilage) and the dorsolaterally placed thyroid lobes were then visualized under binocular dissecting microscope by removal of the glossal muscles and heart posteriorly. This permitted the vascularized glands to be observed clearly in their normal anatomic position ventral to the basal plate of the hyoid (Fig. 2). The cartilage plate with attached thyroids was then dissected free from the lower jaw, removed to filter paper, and the thyroid lobes gently stripped off with watchmaker's forceps and transferred to a stainless steel planchette. Radioactivity in the gland was measured immediately after dissection by a thin mica end-window beta sensitive Geiger-Müller counter under conditions of constant geometry§ for 200-300 seconds. Utilizing this procedure, it was possible to dissect, and measure the radioactivity in, the thyroids of 10-12 tadpoles in about one hour.

Results. A comparison of thyroidal I^{131} and P^{32} uptakes obtained from parallel experiments on normal and TSH treated tadpoles reveals that the larval thyroid concentrates radioiodine much more effectively than radiophosphorus (Tables I and II). Measured under similar conditions, mean percentages of injected radioiodine accumulating in the thyroids of young tadpoles were approximately 10-20 times greater than with P^{32}. This difference in uptake, moreover, was exaggerated throughout normal metamorphosis. The I^{131} collection of thyroids from larvae in advanced stages of development (Table I) increased markedly. The effect was not paralleled with radiophosphorus; the slight increase in P^{32} uptake by the thyroid which occurred in tadpoles with well developed hindlimbs was not statistically significant. Appreciable and significant increase of P^{32} uptake by the thyroid did occur, however, in the climactic stages of metamorphosis (forelimb emergence) although the rise was far less than that with I^{131}. The marked augmentation in uptake of I^{131} by the thyroid with advancing development suggests that the avidity of the gland (per unit mass) for radioiodine may increase progressively in the metamorphic process, and at a greater rate than do growth changes in the gland.

The results obtained with exogenous thyrotrophin are summarized and statistically evaluated in Tables II and III. It is evident that a single injection of TSH in proper dosage in-

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§ Small size of larval thyroid obviates any major difficulty with respect to geometry of radiometric measurement; radioactivity of the dried glands measured 24 hr later in the same planchette is not significantly altered when correction is made for isotopic decay.

TABLE II. Effect of a Single Dose of TSH on Thyroidal P^{32} and I^{131} Accumulation in the Young Tadpole.*

			Mean thyroid P^{32}			Mean thyroid I^{131}		
Exp. No.	Dose TSH (μg)	No. of animals†	% of inj. dose (10^2)	Increase of normal (%)	Level of significance‡	% of inj. dose (10^2)	Increase of normal (%)	Level of significance
1	0	30	.26 ± .04			1.9 ± .17		
	1	30	.31 ± .09	19.2	P n.s.	2.9 ± .30	52.6	$P < .01$
	20	30	.69 ± .05	165.4	$P < .001$	2.8 ± .25	47.4	$P < .01$
	100	13				1.9 ± .28	0	P n.s.
2	0	46	.35 ± .05			3.2 ± .18		
	.5	18	.43 ± .06	22.8	P n.s.			
	2	32	.70 ± .10	100.0	$P < .005$	4.6 ± .48	43.8	$P < .02$
	10	30	1.05 ± .16	200.0	$P < .001$	3.7 ± .32	15.7	P n.s.
	50	15	.78 ± .10	122.8	$P < .005$			

* Animals with hindlimbs ranging from 1-6 mm; 4-8 days from field.

† Divided equally between P^{32} and I^{131} groups.

‡ Calculated from "T" test tables. "P" values in each case are compared against the control ("0" TSH). "P" values $>.05$ are not considered significant (n.s.).

creases radioiodine or radiophosphorus accumulation in the thyroids of tadpoles in early hindlimb stages. The minimal effective dose of hormone required to induce significant uptakes for both radioactive tracers lay at the 1-2 μg level (Table II). The magnitude and range of thyroidal response to increasing doses of thyrotrophin were greater with P^{32} than with I^{131} but in each instance high dose levels resulted in a diminution of thyroidal radioactivity from peak values which fell to approximately normal levels (radioiodine). The effects obtained from continued stimulation of the thyroid by a series of TSH injections were more marked (Table III). The administration of thyrotrophin (U.S.P.) to the stasis tadpole (starved, non-metamorphosing) once daily, over a 4-day period, resulted in significant increases of thyroidal I^{131} uptake ranging from about 250-750% of normal. The proportionality of response to increasing dose of TSH was much greater than that obtained with a single injection of equivalent amounts of the hormone.[‖] The greater increments and wider range of thyroidal radioiodine uptakes produced with chronic TSH treatment were accompanied by somatic changes in the tadpoles indicative of accelerated metamorphosis. In animals receiving the higher doses of thyrotrophin, significant increases in hindlimb extension were noted as well as constriction of the body and head changes characteristic of advanced development. The thyroids of these animals were found to be enlarged and highly vascularized at dissection. It is noteworthy that the minimal effective dose of TSH required to induce a significant increase in thyroidal I^{131} accumulation was of the same order of magnitude with both single and multiple injections. Chronic treatment with high doses of TSH did not reduce radioactivity in the gland, however.

Discussion. The finding of substantial increase in radioiodine accumulation by the thyroid during metamorphosis closely accords with results of previous studies establishing the basic importance of thyroid-pituitary interaction in amphibian development. The growth and histologic manifestations of secretory activity in the thyroid of developing Rana tadpoles have been shown to increase progressively to a maximum, coinciding closely with the appearance of the climax stages of metamorphosis(4-6). These thyroidal changes and their metamorphic sequelae in turn are dependent upon concomitant changes in thyrotrophic hormone secretion from the tadpole's anterior pituitary and can be induced precociously with exogenous TSH(7-10). It is quite likely, therefore, that the enhanced I^{131} uptake by the thyroid in normal metamorphosis is a further expression

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[‖] 1 μg of Parke, Davis preparation (50P4) is equivalent to approximately 2 milliunits of U.S.P. Reference Standard.

TABLE III. Thyroidal Radioiodine Collection in the Stasis Tadpole* after Chronic Treatment with U.S.P. Thyrotrophin.

No. of animals	Mean increase in hindlimb growth (mm)	Dose TSH‡ (U.S.P. units)	Thyroid I^{131}, mean counts per 100 sec.	Mean % increase of normal	Level of significance‖
14	—	0	440 ± 45§	—	—
14	.9	.0002	447 ± 53	1.6	P n.s.
15	1.1	.0012	471 ± 76	7.0	P n.s.
14	1.4	.006	1500 ± 150	240.9	$P < .001$
14	1.7†	.03	3251 ± 499	638.9	$P < .001$
14	2.0†	.15	3793 ± 623	762.0	$P < .001$

* Starved, non-metamorphosing.
† Body changes indicative of advanced metamorphosis.
‡ Total dose given over experimental period (4 days).
§ Mean number of counts ± stand. error of mean.
‖ "P" values $< .001$ are highly significant; "P" n.s. (not significant).

of the increased tempo of thyroid function resulting from augmented titers of circulating endogenous thyrotrophin. It cannot be stated with certainty how much of the radioiodine accumulating in the thyroid reflects increased growth as against enhanced avidity of the gland per unit mass. The small size of the larval thyroid precludes direct weight measurements but from dimensions obtained with camera lucida and from histologic sections the estimated weight of the developing gland ranges from 50 to 200 μg. The concentration of radioiodine in the thyroid during advanced metamorphosis is far greater than can be accounted for by increased size of the gland, and uptakes may actually exceed those described by Bradley(11) for the frog (*Rana pipiens*). A detailed study of thyroidal uptake through development and into post-metamorphic stages is indicated.

The significance of phosphorus uptake in thyroid function (unlike that of iodine which is an essential component of thyroid hormone) is poorly understood. Chemical studies by Borell(14) pointed to a direct relationship between the phosphorus content of the thyroid and the amount of follicular epithelium. Borell and Holmgren(15) and Lamberg(16) for guinea pig and chick respectively, found good correlation between acinar cell height increase and P^{32} uptake in TSH stimulated thyroids. The present study indicates that the augmentation of P^{32} uptake with TSH also holds for the amphibian thyroid. The similarity of the response in representatives of at least three vertebrate classes seems highly significant, and it may well be that phosphorus has special importance as a component of the growing thyroid cell(14). It remains to be shown, however, whether or not the uptake of phosphorus by the thyroid in metamorphosis truly parallels growth of the gland, and whether it relates specifically to colloid or to epithelium. The relationship between iodine and phosphorus metabolism in the hypertrophic gland should also be investigated.

The increases in thyroidal I^{131} and P^{32} uptakes induced with relatively small doses of TSH are of particular interest as regards the problem of thyrotrophin assay. The dose response curves obtained with a single injection of the hormone did not display a range of proportionality which could lead to precise estimation of TSH in blood and pituitary, as now routinely accomplished with microhistometric measurement of thyroid cell height in the stasis tadpole(13,12). Large doses of TSH tended to decrease rather than increase radioactivity in the thyroid. It is conceivable that purging of thyroid hormone from the gland with sharp and vigorous stimulation may induce transformation changes (fluid loss?) in the test animal which temporarily diminishes the amount of radioactive tracer available to the gland. Whatever the cause, the effect was overcome by chronic stimulation of the thyroid with TSH. In fact, thyroidal I^{131} uptakes in stasis tadpoles given a series of TSH injections were sufficiently large over a wider range of dose level to merit further investigation of the response as to its usefulness for thyrotrophin assay. A detailed comparison of histometric and radiometric methods for TSH detection in the stasis tadpole will be considered elsewhere.

Summary and conclusions. 1) The uptake of I^{131} and P^{32} by the developing thyroid of Rana tadpoles has been determined in normal and accelerated metamorphosis. Radioactive measurements of the isolated gland (estimated weight, 50-200 μg) disclose a progressive increase in radioiodine accumulation throughout normal development. The effect is not paralleled with radiophosphorus although significantly increased P^{32} thyroidal uptakes eventually occur in advanced development. Exogenous thyrotrophin augments I^{131} and P^{32} accumulation in the thyroids of young tadpoles. The applicability of the thyroidal response to the bioassay of thyrotrophin is discussed. 2) The enhancement of iodine and phosphorus uptake by the larval thyroid is considered to reflect increased tempo of thyroid function in amphibian development. The fundamental rôle of pituitary-thyroid interaction in anuran metamorphosis is reemphasized.

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Lack of Lipotropic Action by Thiomethyl Derivatives of Glucose.* (22584)

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The fatty infiltration of the livers of rats on low protein diets is generally ascribed to a deficient synthesis of choline as a result of the inadequate supply of biologically transferable ("labile") methyl groups. In methionine, which is the major dietary source of such groups, the methyl is attached to a sulfur atom; and the same type of attachment is present in methionine sulfoxide and in various thetins (dimethylpropio-, dimethyl-, methylethyl thetin, and methionine ethylsulfonium), which have been shown to be efficient methyl donors in the rat(1). It seemed possible that other compounds possessing terminal thiomethyl groups may also be active in this respect. In the present investigation, the lipotropic action of 2 such compounds, methyl-thioglucoside (MTG) ($CH_3 \cdot S \cdot CH_2 \cdot (CHOH)_4 \cdot CH_2OH$) and glucose dimethylmercaptal (GDM) ($(CH_3 \cdot S)_2 \cdot CH \cdot (CHOH)_4 \cdot CH_2OH$), was tested.†

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† The thiomethyl derivatives of glucose were synthesized by Herstein Laboratories, New York City. The author is indebted to the Sugar Research Foundation for the generous gift of these compounds and for their interest in the project.

TABLE I. Average Phospholipid and "Fat" Contents of Liver of Rats on a Low-Protein Diet with or without Supplements.[*]

			Per g of liver		Per liver of 100 g rat	
Dietary supplement[†]	Gain in body wt, g	Liver wt for 100 g rat, g	Phospholipids, mg	"Fat," mg	Phospholipids, mg	"Fat," mg
None (23)	5.99 ± 1.3	5.86 ± .4	24.9 ± 2.0	112.0 ± 15.3	150.3 ± 11.9	675.7 ± 107
Choline (21)	6.77 ± 1.4	4.82[§] ± .3	26.0 ± 2.1	41.7[‡] ± 5.2	121.0[§] ± 8.0	202.1[‡] ± 23
MTG (15)	7.62 ± 2.86	5.73 ± .6	27.6 ± 4.0	132.7 ± 23.5	154.4 ± 22.2	775.8 ± 148
GDM (23)	9.13 ± 1.34	5.58 ± .4	25.0 ± 1.4	108.4 ± 13.2	135.4 ± 6.5	609.1 ± 84

[*] Values preceded by ± are stand. errors of means.

[†] No. of rats in parentheses.

[‡] Significantly different ($P < 0.01$) from corresponding value of group on the unsupplemented diet.

[§] Significantly different ($P < 0.05$) from corresponding value of group on the unsupplemented diet.

Moreover, since choline-deficient livers show a decreased ability to oxidize isotopic fatty acid *in vitro*(2), the possibility that such an effect could be prevented by supplementation of the diet with MTG or GDM was also investigated.

Methods. Male weanling rats (Rockland Farms, initial weight 35-45 g) were kept on an experimental diet (Diet 46, containing casein 8%, sucrose 28%, dextrin 28%, Crisco 28%, Ruffex 2%, cod liver oil 2%, salt mixture (USP XII) 4%). A B vitamin mixture (2) was incorporated in the diet. The rats were housed not more than 6 per cage and were given food and water *ad libitum*. In each experiment one group was maintained on the unsupplemented diet. Other groups received 3 mM of choline chloride, MTG, or GDM, per 100 g of diet, except in one experiment in which the methyl glucose compounds were fed at levels calculated to contain the same number of methyl groups as choline (9 mM of MTG, or 4.5 mM of GDM). After 21 days on the diets, the rats were killed by decapitation. The liver of each animal was weighed and its content of total lipids (weight of the chloroform extract(3)) and lipid phosphorus(4) was determined. The differences between total lipids and total phospholipids (lipid P x 25) are indicated as "fat". Such values, in addition to triglycerides, include cholesterol and other unsaponifiable substances. In the experiments on fatty acid oxidation, homogenates prepared from livers of rats on the supplemented, or unsupplemented, diets were incubated simultaneously in the presence of stearate-1-C^{14}. After 3 hours in air at 37°C, the $C^{14}O_2$ produced was determined. For further details on the procedures, see Artom(2).

Results. For the sake of brevity, only the averages and standard errors of the results obtained on the individual rats are recorded in Table I. Phospholipid and "fat" values have been calculated both per g of tissue and for the whole liver of a 100 g rat: the latter method of expressing the results allows a direct comparison of the values obtained in the various groups, in spite of considerable variations in the size and fat content of the liver. The differences between the averages of the groups on the supplemented diets and those of the control rats on the unsupplemented diet were considered significant, when the ratios of these differences to their standard errors were greater than 2 ($P<0.05$), or 3 ($P<0.01$).

The results, expressed by either method, indicate that choline supplementation of the diet lowered significantly the fat content of the liver. Total phospholipids were also lower, but only when the results were referred to the whole liver of a 100 g rat. On the other hand, it is apparent that the fatty infiltration in the livers of rats receiving MTG or GDM is of the same degree as that seen in the livers of the controls on the unsupplemented diet.

In Table II the results of one of our experiments on the *in vitro* oxidation of stearate-1-C^{14} are recorded. Data on the $C^{14}O_2$ produced during the incubation (expressed as counts per minute per mg N of tissue, as well as per liver of a 100 g rat) indicate that the livers of the rats on the choline-supplemented

TABLE II. $C^{14}O_2$ Production from Stearate-1-C^{14} by Liver Homogenates.*

Rat	Dietary supplement	$C^{14}O_2$ (counts/min.) Per mg N	Per liver of 100 g rat
a	None	121	14,750
b	"	212	25,800
c	Choline	835	70,000
d	"	1000	116,000
e	GDM	256	32,500
f	"	163	17,000

* Each value is avg of data obtained on duplicate flasks containing the same homogenate. Each flask contained, in a total volume of 6 ml of Ringer-phosphate without Ca and Mg (pH 7.4), 0.375 g of homogenized liver, 12 μmoles of adenosine triphosphate, 3 mg each of penicillin and dihydrostreptomycin and 1 μmole (= 1 μC) of stearate-1-C^{14}.

diet oxidized isotopic stearate at a much higher rate than the livers of the control rats. On the other hand, $C^{14}O_2$ produced by the livers of the animals receiving MTG or GDM was about the same as that produced by livers of rats on the unsupplemented diet.

It is now established that the transfer of the methyl group of methionine must be preceded by the conversion of the free amino acid into an active form, S-adenosylmethionine(5). It seems probable that transmethylation from the thiomethyl groups present in other compounds should require a similar activation of the sulfur to the "onium" level. The lack of specific enzymes for the conversion of the glucose derivatives which we have tested, into active forms might perhaps explain their inability to act as methyl donors in the organism.

Summary. Supplementation of a high fat, low-protein diet with two thiomethyl derivatives of glucose (glucose dimethylmercaptal and methyl-thioglucoside) did not prevent the fatty infiltration of the liver, and did not enhance the ability of the tissue to oxidize fatty acid added *in vitro*.

The continued interest and helpful suggestions of Dr. C. Artom are gratefully acknowledged.

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Free Electrophoresis of Lipoproteins. (22585)

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There are comparatively few reports on serum lipoprotein investigations by moving boundary electrophoresis(1,2), because of the difficulty inherent in the interpretation of the patterns due to the low density of the lipoproteins(3) and their possible transformations during dialysis. Paper electrophoresis (4,5) has yielded some information on these substances but this method too, has its shortcomings because of the adsorption of lipid or lipoproteins and their stains on the paper(6). In addition, differences have been found between moving boundary and paper electrophoresis for α_2-, β-, and γ-globulins(7).

In the present investigation, advantage is taken of the ultracentrifugal technic for isolation of the low density lipoproteins(8). The characteristics of the latter as revealed by moving boundary electrophoresis were studied and it is believed that this procedure can be used profitably in comparative lipoprotein investigations.

Methods. Blood (80 ml) was collected from each of 5 healthy male medical students,

TABLE I. Chemical Analysis of Fractions Obtained in Ultracentrifugal Separation.

	Total protein, g %	Phospholipid, mg %	Total cholesterol, mg %
Whole serum	6.85-7.21	194.5-245.0	224- 258
Lipoprotein concentrate	1.52-1.23	580 -720	920-1040
Lipoprotein-poor solution	3.13-3.92	135 -144	64- 67

was allowed to clot at room temperature for 2 hours, after which the serum was separated by centrifugation. A portion of this serum was used for electrophoretic analysis. The rest was diluted with an aqueous sodium chloride solution to give a final solution density of 1.0631 and centrifuged at 30,000 rpm (16°C) for 18 hours in a Spinco Model L ultracentrifuge. The top layers containing the low density lipoproteins were taken off and combined and the bottom fractions were also combined and saved. The former is designated below as the lipoprotein concentrate and the latter as lipoprotein-poor serum solu-

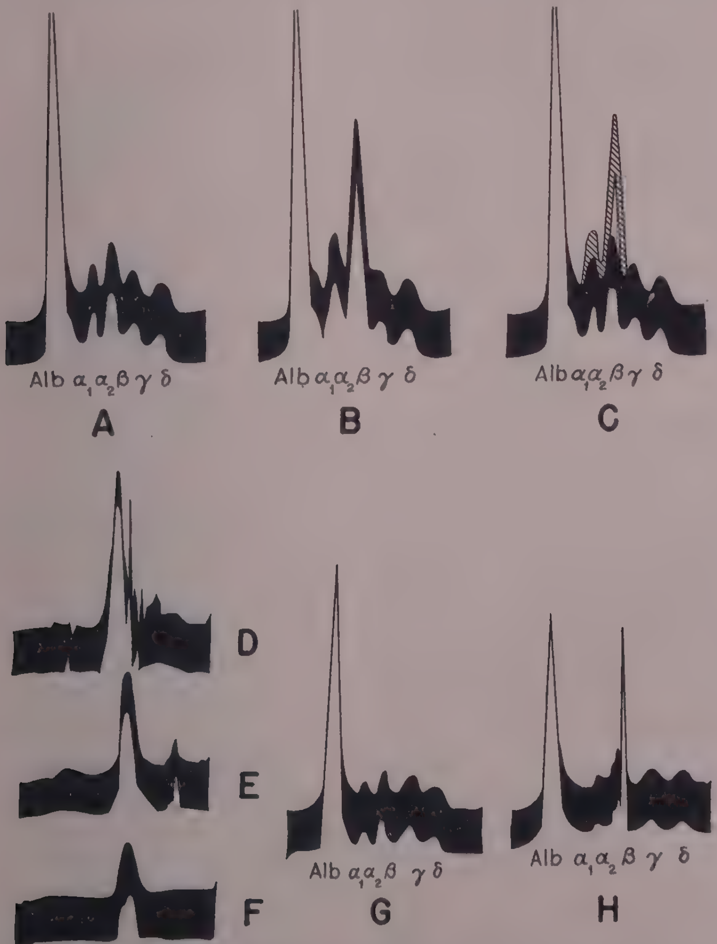

FIG. 1. Representative electrophoretic patterns obtained in these experiments. *A* is normal serum. *B* is normal serum plus 0.5 ml lipoprotein concentrate. *C* shows *B* superimposed on *A* (crosshatched area is effect of added lipoprotein). *D*, *E* and *F* are patterns obtained with solutions of lipoprotein concentrate 1.5, 1.0 and 0.5 ml, respectively, in a total volume of 3 ml. *G* is lipoprotein-poor serum and *H* shows effect of adding 0.5 ml of lipoprotein concentrate to *G*.

tion. Table I gives the analyses on the 3 fractions. For electrophoretic analysis, 1 ml of clear serum was diluted with 2 ml veronal buffer pH 8.6 and ionic strength 0.1. The same buffer was used for all fractions. The diluted serum was dialyzed for 30 hours against 450 ml of the buffer at 4°C. Analyses were made in the Perkin-Elmer electrophoresis apparatus model 38. The length of the runs was 4,200 sec. (1°C) and the patterns were enlarged and calculated from the areas under the curves. A representative pattern is shown in Fig. 1 A. Lipoprotein concentrates, 1.5, 1.0 and 0.5 ml were diluted to 3 ml with the buffer and dialyzed for 48 hours against the same buffer. Two changes of the buffer were made. Electrophoretic runs were carried out for 4,800 sec. (Fig. 1 D, E, F). Lipoproteins-poor solution was dialyzed against veronal buffer for 72 hours with 3 changes of the buffer. Runs were made for 4,200 sec. (Fig. 1 G). .1, .25, .40, .50, and 1.0 ml of lipoprotein concentrate was added to 1.0 ml of normal serum and these were diluted with 1.9, 1.75, 1.6, 1.5 and 1 ml respectively, of buffer. These were dialyzed twice against 450 ml of buffer for 48 hours. Electrophoretic runs were made for 4,200 sec. (Fig. 1 B and C)

Results. Fig. 1 D, E and F show the electrophoretic pattern of the lipoprotein concentrate at different dilutions. Addition of this material to normal serum results in an increase of the β-globulin and a concomitant decrease in albumen (Fig. 1 B, C and Fig. 2). This excess lipoprotein causes no major alteration or disturbance of the general pattern and the individual peaks are easily discernible. It might, therefore, be inferred that the pattern disturbance seen in lipemic serum (9, 10) is not due to the low density lipoprotein but to some other substances.

On the other hand, removal of the low density lipoproteins results in a decrease in the β-globulin peak and to a smaller extent in the α_2- and γ-globulin peaks (Fig. 1 G). When lipoprotein concentrate is added to this solution it gives almost the same results as when it is added to whole serum (Fig. 1 H). The only difference is that the α_2-globulin does not change as it does with normal serum.

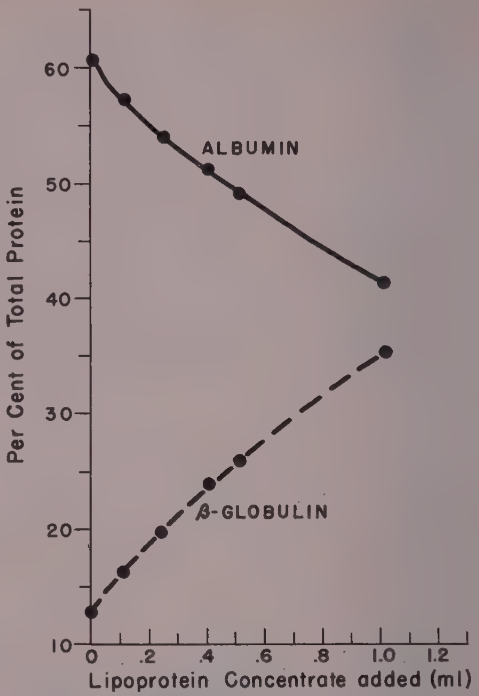

FIG. 2. Effect of adding lipoprotein concentrate to normal serum as calculated from electrophoretic patterns.

It is realized that lipoproteins produce various boundary artifacts in free electrophoresis; however, the technic here suggested can be used to study various changes in lipoproteins during metabolic studies. The effect of various agents on the electrophoretic properties can be observed on a macro scale, and clinical comparisons of lipoproteins under different conditions can be made.

Summary. Analyses by free electrophoresis have been made on the low density lipoproteins obtained by centrifugation of serum at a solution density of 1.063. At pH 8.6 the lipoproteins give a clear electrophoretic pattern showing concentration dependence, but no change in mobility with increase in concentration. Addition of this material to normal serum or lipoprotein-poor serum results primarily in an increase in the β-globulin peak. On the other hand, when the lipoproteins are removed from the serum a significant decrease in this peak is observed. This method is suggested for possible metabolic studies with these lipoproteins.

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Daily Changes in 5-Hydroxytryptamine Concentration in Mouse Brain.*†

(22586)

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The status of our knowledge on 24-hour periodicity has been reviewed from neurophysiologic(1) and endocrinologic points of view(2). Almost certainly, 24-hour periodic body function involves a nervous-endocrine-metabolic sequence(3), and in this connection the behavior of neurohumors around the 24-hour time scale appears to be of interest. Earlier work from this laboratory has dealt with the effects of 5-hydroxy-tryptamine (5-HTA) upon the number of circulating eosinophils(4), and thus, perhaps, with the effects of this neurohumoral agent upon adrenal mechanisms of physiologic 24-hour periodicity. This report is concerned with the daily changes in the concentration in mouse brain of 5-HTA itself. From thorough reviews of the literature on 5-HTA, made by Erspamer (5,6), it would appear that this problem has not previously been considered.

Materials and methods. Seven series of determinations were made on ZBC‡ mice, male or female, 5 or 7 weeks of age. Purina Fox Chow and tap water were available to all of the mice since weaning and until sacrifice. For at least 7 days prior to sacrifice, the mice were singly housed in a room maintained at 78 ± 2°F, and illuminated by artificial light only. A clock controlled switch turned the lights on at 06:00 and off at 18:00.

In a given series, groups composed each of 25 mice were used during certain periods of day (specified under results). During any one period of day, individual mice were decapitated, a mid-line incision was then made through the skull with small, pointed scissors, the skull divided, and the entire brain was removed and weighed to the nearest milligram on a torsion balance. Immediately after weighing, the brain was transferred to a previously cooled mortar containing 95% acetone (20 ml/g, as suggested by Amin *et al.* (7)) and crushed with a pestle. The time elapsed between decapitation and the beginning of extraction did not exceed 1½ minutes.

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† Grateful acknowledgement is due to Miss M. Cameron for her excellent technical assistance.

‡ ZBC mice are obtained by mating F_1 hybrid females of several stocks to a male of the Z or Zb stock.

After a minimum of 24 hours, the acetone extract was filtered off, and the brain residue was re-extracted with an equal volume of cold 95% acetone. The 2 extracts were combined and evaporated to dryness at 37°C *in vacuo*. The dry residue was taken up in 3 ml of 0.1 N HCl, and its 5-HTA content determined spectrophotometrically according to the method of Udenfriend *et al.*(8), with only slight changes made in order to adapt the procedure to available equipment. Each of the values for brain 5-HTA concentration here discussed is based upon the pooled extract of 25 mouse brains. The specificity of the method has been discussed by Udenfriend (8), and the possible interference by materials other than 5-HTA has not been evaluated herein. Methodologically, a check of sensitivity was first undertaken. Quantities of 6 μg of synthetic 5-HTA in water solution could reliably be determined, in close agreement with Udenfriend's report. Next, pools of mouse brains were studied in order to obtain 5-HTA values of comparable magnitude. The choice of as many as 25 mouse brains as source material for one extract was made in view of earlier work on brains from several species(7,9,10), in the light of which the necessity of such a pool was anticipated. Third, pools with and without added 5-HTA were extracted and analyzed, and in two such studies about 90% of the added 5-HTA was recovered.

TABLE I. 5-HTA Concentration in Mouse Brain* at Certain Times of Day.

	5-HTA, μg/g (wet wt)						
	Series No.						Mean
Time of day	1	2	3	4	5	6	± S.E.
8:30- 9:30			.76	.65		.89	.81 ± .03
9:30-10:30						.82	
10:30-11:30	.73	.70			.89	1.05	
12:00-13:00		.72	.78	.74	.90	.90	
16:30-17:30			.62	.55	.87	.86	.69 ± .03
17:30-18:30						.74	
18:30-19:30	.53	.59				.73	
19:30-20:30		.61			.80	.72	
20:30-21:30			.60	.73			

* Values describe pools consisting each of 25 brains from ZBC-Bittner mice.

Males were used for series 1-5, females for series 6; mice 5 wk of age were used for series 1-3, mice 7 wk of age were used for series 4-6.

Results. Among 7 brain pools, obtained in an exploratory series at 4-hour intervals from 08:30 of one day to 08:30 of the next, the highest 5-HTA value was found at 12:30 and the lowest at 16:30. Subsequently, the study was focused upon the detection of changes which may occur around these time points. Pertinent data from 6 series are shown in Table I, which reveals that, on the average, the concentration of 5-HTA in brain was higher before and around noon than at or after 16:30. The corresponding difference of .12 ± .04 was analyzed by the use of Student's t and was significant at 2% ($t = 2.76$; $P = .011$). It also is pertinent that similar daily changes may be seen in Table I for males of 2 age groups (series 1-5) and for females (series 6). But this change in 5-HTA concentration as a function of time of day is more readily apparent from Fig. 1; the daily change stands out clearly in data expressed as per cent of series mean. For comparison of 5-HTA concentrations at several ages, data are available in this study on males 5, 7, and 16 weeks of age. The mean 5-HTA concentration of 5 pools describing mice 5 weeks of age was .74 μg/g (with SE = .01 μg/g), while the corresponding values of 2 pools describing mice 7 weeks of age were .65 and .74, and that for one pool describing mice 16 weeks of age was .82. Further observations will be needed to clarify the question of possible age trends in brain 5-HTA concentration.

Table II compares the 5-HTA content in brain pools from mice of the two sexes, but of roughly comparable age and stock. Without generalizing beyond the scope of the limited sample size employed, it would appear that the concentration of 5-HTA may be slightly higher in females than in males. The analysis of the sex difference of .15 ± .07 yielded a $t = 2.03$ and a P of .07.

Discussion. Previous work on brain 5-HTA dealt with the central nervous system as a whole, as well as with defined parts thereof. By means of bioassay, Twarog and Page(9) have reported concentrations of .1 and .36 μg/g for whole dog brain, and a concentration of .24 μg/g in rat brain. Also by bioassay, Amin, Crawford, and Gaddum(7) have thor-

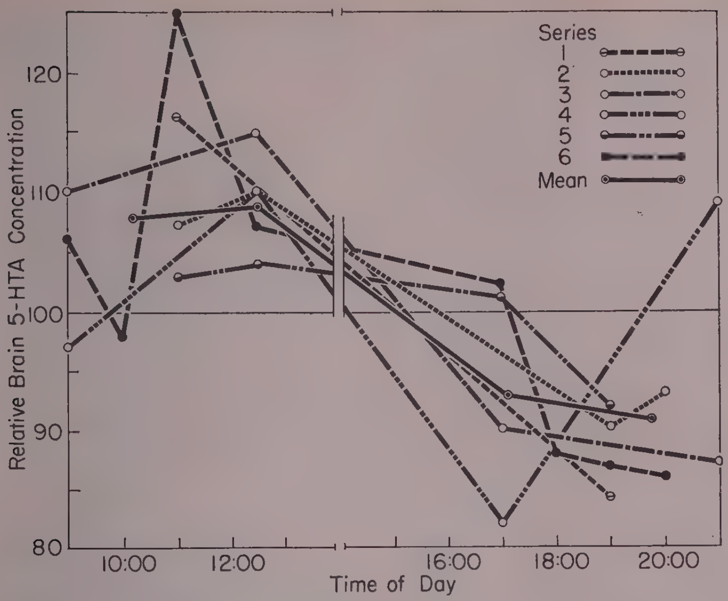

FIG. 1. Daily changes in 5-HTA concentration in mouse brain expressed as % of mean for each series.

oughly analyzed the 5-HTA content in defined parts of the brain of the dog. More recently, Pletscher, Shore, and Brodie(10), using a chemical method, have reported a "normal average" of .54 μg/g in rabbit brain. Using the same chemical method, Bogdanski and Udenfriend(11) have reported concentrations of between .07 and 1.75 μg/g for various parts of dog brain, and between .30 and 1.78 μg/g for various parts of cat brain. In both instances, the highest concentrations were found in the hypothalamus. The data of this report on mouse brains are roughly of the same order of magnitude. Moreover, if, as seems likely, the apparent 5-HTA in brain determined chemically in this study is identical with authentic 5-HTA, several suggestions can be made on the basis of the data presented herein.

1. It would appear that the 5-HTA concentration in mouse brain constitutes a 24-hour periodic variable. This finding is clearly visualized in Fig. 1, in which the data of each time series are expressed as % of series mean, *i.e.*, as relative 5-HTA concentrations. Even though the absolute values at the time of daily peak concentration appear to exhibit a sex difference (being higher in females than in males studied at the same time of day, Table II), both sexes exhibit similar "within day" changes (compare series 6 with series 1-5, Fig. 1).

2. The temporal placement within the 24-hour period of the daily decrease in brain 5-HTA concentration is of added interest. Under the conditions of this study, this decrease precedes the usual period of onset of increased body activity, which in the nocturnal mouse occurs around the beginning of the dark period(12). It may be pertinent that daily adrenal activation in mice does occur during roughly the same period(2,13) and also that Bertelli, Cantone, and Martini have implicated 5-HTA as a mechanism of ACTH

TABLE II. Sex Comparison* of Brain 5-HTA in ZBC Mice, 5 and 7 Weeks of Age.

Sex of mice	No. of pools†	5-HTA, μg/g, mean ± S.E.
♂	6	.78 ± .05
♀	5	.93 ± .04

* Based upon data obtained during daily time period of apparent peak concentration (Table I and text).

† Each pool consisting of 25 mouse brains.

release from the anterior pituitary(14).

3. (And also apparent from Fig. 1) the extent of daily change in brain 5-HTA concentration is relatively small as compared with the amplitude of 24-hour changes observed under standardized circumstances for some other body functions of rodents(2,15). The suggestion by Pletscher, Shore, and Brodie(10) that 5-HTA exists mainly in a bound form comes to mind in this connection. Before this assumption is validated, the possibility that those changes noted herein may reflect primarily changes in free 5-HTA can hardly be discussed. It also must be remembered that 5-HTA behavior of the entire brain was studied herein. Thus, drastic changes in certain parts of the brain, like the hypothalamus, may be obscured if other parts of the brain do not vary at the same time in the same direction. Analysis of these problems must await the study with more sensitive procedures(8) of the behavior along the 24-hour time scale of 5-HTA concentration in various areas of the brain.

Summary. ZBC mice, male or female, 5 or 7 weeks of age, were studied under conditions standardized for evaluation of 24-hour periodicity. A small but significant decrease in 5-HTA concentration of brain pools was noted prior to usual time of arousal. A sex difference in brain 5-HTA concentration also was recorded, the females exhibiting slightly higher values.

The authors are indebted to Dr. M. Speeter, Upjohn Co., and to Dr. R. K. Richards, Abbott Co., for generous gifts of 5-HTA, used as standards.

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Metal Chelates as Therapeutic and Detoxifying Agents in Gas Gangrene.* (22587)

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The ability of ethylenediamine tetraacetic acid (EDTA) to protect mice against a lethal dose of *Clostridium perfringens* Type A toxin has been noted(1). It was shown that this protection was due to the metal chelating ability of EDTA inasmuch as it was reversed by Zn^{++}, Co^{++} and Mn^{++}. In those experiments the toxin and EDTA were mixed together before injection but it appeared that EDTA could also protect when given independently of the toxin. In relatively large doses EDTA is toxic for animals because it induces hypocalcemia, and thus large amounts cannot be administered safely. Although Ca^{++} activates the principal lethal component of this toxin, a lecithinase, it was demonstrated that it did not reverse the protective effect of EDTA. This observation suggested that the calcium chelate of EDTA (Ca-

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* Aided by a grant from the Purdue Research Foundation and Grant G-3444 of the National Heart Institute, National Institutes of Health.

TABLE I. Protective Effect of CaEDTA in Mice Injected with *Cl. perfringens* Toxin.

Conc. CaEDTA inj. (%)	No. survivors/ No. animals inj.
5	10/10
2.5	8/10
1	7/10
.33	3/10
0	0/10

EDTA), which is non-toxic, may be of value in protecting against the toxin, and also perhaps as a therapeutic agent in gas gangrene. *Cl. perfringens* is the organism most frequently involved in gas gangrene and its virulence is thought to be due to a battery of enzymes which aid it in invading tissues. It was hypothesized that if CaEDTA inhibited all, or some of these enzymes, the invasiveness of the organisms would be curbed and the infection would be stopped. In this report evidence that CaEDTA does protect animals against a toxic culture filtrate of *Cl. perfringens* and experimental gas gangrene is presented.

Procedures and results: *Protection against a toxic culture filtrate*: It was determined that 0.5 cc of a 1:4 dilution of a toxic culture filtrate of *Cl. perfringens* Type A[†] contained 2 M.L.D. when injected intracutaneously (I.C.) into 20-25 g white mice. All mice died within 24 hours with this dose of toxin. The CaEDTA used was a purified preparation dispensed for the treatment of lead poisoning.[‡] All dilutions of the CaEDTA and toxin, and in later experiments the cultures and EDTA, were made in sterile 0.15 M NaCl. Five-tenth ml of toxin was injected I.C. into one side of the abdominal surface and at the same time 0.5 ml of the CaEDTA solution was injected I.C. into the abdominal surface on the other side of the mouse. The results are given in Table I. The survivors given toxin plus 5% or 2.5% CaEDTA manifested normal activity and no sign of illness, while those surviving with 1% and 0.3% CaEDTA were ill for a few days before resuming their normal activity.

Protection against experimental gas gangrene. These experiments were initiated on an exploratory basis but the results are so definitive that they are deemed worthy of being reported at this time. Lack of animal facilities prevented the use of larger numbers of animals, thus there is a variation in the number of animals in some experimental groups and questions relating to the optimum therapeutic utilization of CaEDTA remain to be answered. A strain of *Cl. perfringens* isolated from a patient with gas gangrene[§] was grown in a medium composed of 3% Trypticase Soy Broth and 1% Proteose Peptone #2. An infection was initiated by injecting a dilution of a 5 hr culture into the thigh muscle of a guinea pig. CaEDTA was administered by subcutaneous injection of a 5% solution into the axillary region. The dose was 30 mg/100 g and was equally divided between each axilla. The initial injection of CaEDTA was made at the same time the culture dilution was injected. When more than one injection of CaEDTA is recorded they are each at this dosage level at approximately 12-hour intervals. In some experiments the culture was diluted either in 1% CaEDTA or 1% EDTA[‖] and the volume of these suspensions injected was the same as that recorded for the culture dilution. These latter solutions were adjusted to pH 7.3 before the cultures were diluted in them and it was determined that they were not bactericidal in these concentrations. The different groups of animals in an individual experiment were injected with aliquots of the same culture dilution within the time interval necessary to carry out the injections. The results of the experiments are recorded in Table II.

Comments. In Exp. I, the animals in Groups B and D were slightly ill, except for the one fatality which became ill on the third day. Except for Group D in Exp. III, none of the treated animals in Exp. II and III

[†] Kindly supplied by Lederle Laboratories Division, American Cyanamid Co.

[‡] Sold under trade name of Versenate. It is the calcium disodium salt of EDTA. Kindly supplied by Riker Laboratories, Inc., Los Angeles, Calif.

[§] "Geiger" strain. Obtained from Laboratory of Dr. John Maclennan.

[‖] Disodium Dihydrogen Versene, analytical reagent. Kindly supplied by Dow Chemical Co.

TABLE II. Effect of CaEDTA and EDTA on Experimental Gas Gangrene.

Exp.		Dilution of culture inj.*	Treatment	No. animals in group§	Time of death (No. dead in hr)					No. survivors	Extent of lesion of survivors‖ (No. in each category)				
					24	48	72	96	120		A	B	C	D	E
I	A	1:10	None	2	2	—	—	—	—	0	—	—	—	—	—
	B	”	5 inj. CaEDTA	4	0	0	0	0	1	3	0	0	3	0	0
	C	1:100	None	2	2	—	—	—	—	0	—	—	—	—	—
	D	”	5 inj. CaEDTA	4	0	0	0	0	0	4	0	0	4	—	—
II	A	1:50	None	8	2	1	1	1	0	3‡	0	0	0	0	3
	B	”	3 inj. CaEDTA	8	0	0	0	0	0	8	4	3	1	0	0
	C	”	1 ” ”	6	0	0	0	0	0	6	3	0	3	0	0
	D	”	Culture dil. in EDTA	5	0	0	0	0	0	5	1	1	3	0	0
III	A	1:20	None	10	5	4	1	—	—	0	—	—	—	—	—
	B	”	1 inj. CaEDTA†	10	0	0	0	0	0	10	7	2	1	0	0
	C	”	Culture dil. in EDTA	4	0	0	0	0	0	4	1	0	0	1	2
	D	”	” ” ” CaEDTA	4	0	0	0	1	1	2	0	0	0	0	2

* 0.5 ml in Exp. I; 0.25 cc in Exp. II and III.

† Three animals given 2nd inj.

‡ Unlike other survivors, who were recovering, these had gaping holes in their sides and were moribund (see text).

§ Wt range of animals: Exp. I, 750-900 g; Exp. II and III, 300-500 g.

‖ Extent of lesion: Lesion recorded is maximum that developed (see text). A. Slight or no swelling at inj. site. B. Definite swelling at inj. site. C. Swelling involves most of thigh. D. Swelling involves rear quadrant. E. As in D, but with gas spreading up side of body.

became ill, in spite of the lesions that developed in many of them. The lesions of treated animals recorded as A, B and C in Table II developed to that extent within the first 24 hours after injection, and started to subside after that time. At the end of the 5-day experimental period most of them were healed. The lesions of treated animals recorded as D and E developed to that extent in the course of about 4 days, then started to subside.

Discussion. Inasmuch as the toxins of *Cl. perfringens* are thought to be responsible for the ability of the organism to invade tissues and cause death, the fact that CaEDTA protects against a lethal dose of the toxin suggests that it protects against experimental infection by inhibition of one or more of the toxins that are produced. Although only a small number of animals were involved, when the organisms were suspended in either CaEDTA or EDTA the latter appeared to have a greater protective effect. These results do not detract from the effectiveness of CaEDTA when given systemically in view of the marked differences in dosage used with the 2 methods of administration. They do suggest, however, that the optimum therapeutic approach may be to use CaEDTA systemically and EDTA locally. It was previously suggested (1) that CaEDTA protects against the toxin because Zn^{++}, Co^{++} and Mn^{++}, either of which are necessary for the action of the toxin in the presence of CaEDTA, are preferentially complexed over Ca^{++}. If this hypothesis obtains, the protection afforded by the CaEDTA when administered systemically suggests that the "free" metallic ions in the body that are preferentially complexed over Ca^{++} are complexed and prevented from playing an active role in the body. Inasmuch as these ions may play a role in the growth of some microorganisms or the activity of the toxins produced by them, the use of CaEDTA and related chelating agents may be of value in other infectious diseases. Also, inasmuch as the activity of some drugs, such as salicylic acid(2) and isoniazid(3), is thought to involve chelation, other chelating agents such as CaEDTA may exert a synergistic action by controlling the amount of "free" metallic ions in the body.

Summary. It has been demonstrated that CaEDTA can protect mice against a lethal dose of *Cl. perfringens* toxin, and guinea pigs

against experimental gas gangrene.

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Responses in Molt and Lay of Fowl to Progestins and Gonadotrophins.* (22588)

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The effects of progesterone in initiating proliferation of the resting feather papilla with consequent molt of the old plumage is well established(1), but there is as yet no clear picture of the point of attack of the steroid in this process. Local stimulation is entirely conceivable in view of the importance of the mesodermal component in feather regeneration. The experimental demonstration, however, has not proven entirely satisfactory(2), although the technics employed may later be found to have been inadequate. Alternatively, progesterone could be thought to act through its effects in the pituitary, the interruption of lay which follows its administration being especially suggestive in this regard. These alternatives were further investigated. Laying hens were treated with compounds other than progesterone having expected progestational activity in one series of experiments. Complementary experiments on possible interference of gonadotrophins or prolactin with the known response to progesterone were carried out simultaneously.

Methods. The hens used belonged to a strain of New Hampshires maintained at the University of Maryland Poultry Farm. They were in their first year of lay and had completed the normal autumnal molt when brought into the laboratory. Each bird was placed in a single cage and individual egg records were kept for over a month prior to treatment. Distribution of the birds to the experimental groups was made on the basis of these records. Individual egg records were continued during the period of treatment and for about 6 weeks thereafter. For tabular representation, weekly production per group was used. Date of molt was arbitrarily set as the day when all birds in the group had commenced a regular daily shedding of body plumage. Duration of molt was counted to the day when the entire group had ceased daily feather loss. No attempt was made to record feather number per hen but notations were made of noticeable differences in individual intensities of shedding. One control group served for all the concurrent experimental series. Testosterone propionate (TP), 50 mg/ml and desoxycorticosterone acetate (DCA), 10 mg/ml were dissolved in 8 parts sesame oil and 2 parts benzyl alcohol. Cortisone acetate (CA) was suspended in the same vehicle, 10 mg/ml. Progesterone (Pg.) was administered as a preparation having slow absorption from local depots.† Pregnant mares' serum (PMS) was Cutter's "Serum Gonadin." Follicle stimulating hormone (FSH), luteinizing hormone (LH) and prolactin (LTH) were Armour's‡ preparations.

Results. Series I. TP and DCA were injected as multiples of the Pg. dosage, corresponding roughly to the respective ratios given by Hooker and Forbes(3) for systemic administration in the mouse. Cortisone acetate was injected as the weight equivalent of the DCA dosage. Details of administration

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† Repositol Progesterone, Pitman-Moore Co.

‡ The FSH, LH and LTH preparations were most kindly furnished by Dr. I. M. Bunding, Armour and Co., Chicago, Ill.

TABLE I. Effect of Compounds with Progestational Activity and of Cortisone Acetate upon Lay and Molt in Hens. Progesterone was given as a single intramuscular injection Jan. 9. TP, DCA and cortisone acetate (CA) were injected subcutaneously daily at the indicated levels Jan. 9-14, and 16-21. Controls were untreated. Onset of molt is counted in days from day 1 of treatment; duration, days.

Treatment, mg	No. of eggs laid by groups of 5 birds each 12/27	1/3	1/10	1/17	1/24	1/31	2/7	2/14	Molt Onset	Molt Duration
Controls	24	25	20	18*	20	22	20	21	—	0
Pg. 20	24	22	2	11†	9	8	12	14	16	10
TP 50	23	22	3	2	0	0	0	0	26	35
DCA 10	24	26	3	0	0	1	8	21	12	19
CA 10	23	23	21	24	24	23	19	20	—	0

* One bird became diseased and ceased lay. † One bird died.

and effects in reproduction and molt are listed in Table I. DCA and TP both suppressed lay and there was a subsequent molt. However, DCA acted more rapidly with respect to both processes and for shorter periods. CA was wholly inactive; the records of this group show that daily handling of the hens was not a factor in the results obtained with other agents.

Series II. Gonadotrophins were given in 10-fold difference, singly and in combination with a constant Pg. dose. Details of administration and effects on egg production and molt are listed in Table II. PMS, 20 r.u., was ineffective on rate of lay; with the addition of Pg., lay ceased at once. A gradual diminution of egg number, more marked with FSH, followed the injection of FSH, 1.0 mg or of LH, 0.5 mg; the addition of Pg. brought a prompt interruption. When the gonadotrophins were administered at the higher levels, the presence of Pg. brought only a slight augmentation in the early period of response. Molt occurred wherever lay ceased.

The FSH treated hens were conspicuous for the appearance of soft-shelled eggs at 1.0 mg and of 2 soft shell eggs upon the same day at both dosages of FSH plus Pg. These observations of premature and multiple ovulations are interesting although admittedly incomplete in absence of autopsies which were incompatible with records of molt.

LTH at 0.5 and 5.0 mg differed markedly from the preceding. Lay was practically unaffected with the low dosage; with the higher level, it was somewhat diminished, but only for the period of treatment. The effect of Pg. with LTH became apparent only at that same period where it further depressed egg number. At no time, however, was ovulation entirely interrupted and within about a week all 4 groups of hens returned to normal lay. LTH

TABLE II. Effect of Anterior Pituitary Gonadotrophins and of Pregnant Mares' Serum, Singly and in Combination with Progesterone, upon Lay in Hens. PMS, FSH, LH and LTH were injected subcutaneously daily into 3 birds at indicated levels Jan. 9-14, and 16-21. Figures in parentheses are numbers of eggs laid by 3 birds upon identical treatment but which in addition received a single intramuscular injection of Pg., Jan. 9. Egg number of 3 birds receiving Pg. alone are entered in bottom line. Onset of molt is counted in days from day 1 of treatment; duration, days. Entries in parentheses apply as in records of lay.

Treatment		Egg No. 12/27	1/3	1/10	1/17	1/24	1/31	2/7	2/14	Molt Onset	Molt Duration
PMS	20 r.u.	12(16)	12(14)	15(0)	16(0)	14(0)	12 (0)	10 (0)	13 (0)	—(12)	0(38)
	200	17(14)	15(13)	5(0)	2(1)	0(0)	0 (0)	0 (0)	0 (2)	19(12)	28(38)
FSH	1.0 mg	16(13)	15(13)	5(5)	2(2)	1(0)	0 (0)	0 (0)	2 (0)	15(14)	19(23)
	10.0	14(15)	14(15)	9(7)	4(4)	0(0)	0 (0)	0 (0)	0 (0)	13(11)	28(25)
LH	.5	12(16)	13(14)	15(1)	16(4)	10(0)	8 (0)	8 (0)	9 (0)	—(16)	0(28)
	5.0	13(16)	15(14)	5(2)	0(0)	0(0)	0 (0)	0 (0)	0 (0)	16(13)	29(31)
LTH	.5	15(11)	14(13)	14(4)	12(1)	15(8)	16(14)	17(13)	17(15)	—(14)	0 (5)
	5.0	15(14)	15(14)	11(2)	4(1)	7(4)	13(13)	13(15)	16(14)	6(14)	18 (7)
Pg.	20	14	13	0	3	5	4	7	11	16	10

thus differs sharply from PMS, FSH and LH in offsetting the effect of Pg. rather than augmenting it as did these gonadotrophins. Furthermore, molt appeared at the 5.0 mg level even though lay had not been wholly suppressed.

Discussion. The data presented here show that an interrupted reproductive phase does not necessarily lead to immediate activation of the papilla (TP, Table I) and conversely, that molt may occur with continued lay (LTH, 5 mg; Table II). The present records of molt take the shedding of the standing feather as measure of onset. Actually, the first events in the papilla occur about a week to 10 days sooner since approximately that time is required for the formation of the underlying feather that ultimately expels its predecessor. This activation occurred early in the periods of treatment, remarkably so with LTH, with the one significant exception of TP treated hens. On the supposition that progestins may directly stimulate the papilla, the delayed response of the TP group could suggest a transformation of part of the androgen to another, progestationally effective compound as has been suggested by Hooker and Forbes(3) in the mouse. However, Layne and Common(4) describe a more rapid onset of molt, toward the end of 10 days of treatment with TP, 5.0 mg/day. These tests were run on immature pullets, 59 days of age, perhaps explaining the difference. The absence of any reaction with cortisone acetate is not surprising since Hertz and Tullner(5) have recently pointed out its lack of progestational activity in the rabbit.

Summary. Progesterone, appropriately administered, has the capacity to a) induce proliferation of the feather papilla with consequent molt, and b) interrupt a laying period. Several compounds with known or suspected progestational activity were tested to ascertain their effectiveness in these respects. Desoxycorticosterone acetate interrupted lay; molt was initiated soon thereafter. Testosterone propionate similarly interrupted lay but molt was evident only after considerable delay. Cortisone acetate was ineffective in either process. Other experiments examined the action of several gonadotrophins given in 10-fold difference of dosage, alone and in conjunction with a constant progesterone dosage. Pregnant mares' serum, follicle stimulating hormone, or luteinizing hormone in adequate dosage diminished or interrupted lay for 3 weeks or more beyond the actual time of treatment. Progesterone augmented all such effects. Molt occurred if lay ceased. Prolactin affected lay very slightly and counteracted interference by progesterone. Molt occurred very early with prolactin alone and was brief and light with progesterone given simultaneously. At the higher dosage level, some molt appeared even though lay was not entirely suppressed.

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Inhibition of P^{32}-Orthophosphate Exchange by Sodium Fluoride in Erythrocytes from Patients with Hereditary Spherocytosis.* (22589)

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To elucidate the mechanism underlying the abnormal metabolic behavior of inorganic phosphate previously demonstrated(1,2) in red cells from patients with hereditary spherocytosis (HS), experiments were carried out to determine whether any difference existed in the effect of fluoride on the orthophosphate-P^{32} exchange reaction between the extra- and intra-cellular compartments. The rate of disappearance of orthophosphate-P^{32} from the plasma at 37°C was used as an index of this isotope exchange reaction(3). As illustrated in Fig. 1, rbc from HS patients are more sensitive to fluoride than normal rbc with respect to the orthophosphate-P^{32} exchange reaction. Results obtained with blood from splenectomized patients did not differ significantly from those obtained with blood from non-splenectomized patients. Reticulocytosis in the latter group did not appear to affect the results significantly.

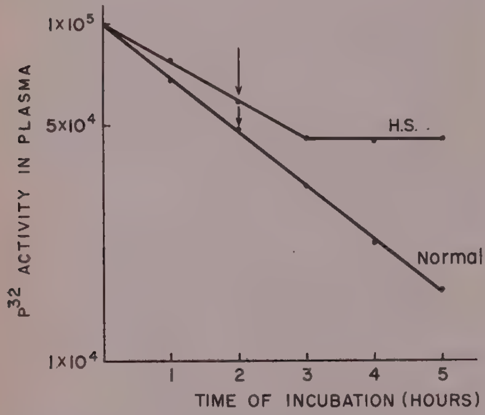

FIG. 1. Orthophosphate-P^{32} exchange by normal and HS red cells in presence of 10^{-3} M NaF; time of addition of NaF indicated by the arrow. P^{32} activity in plasma (ordinate plotted logarithmically) expressed as counts/min. (c.p.m.)/ml of plasma corrected to an initial standard addition of 10^5 c.p.m./ml of plasma. Results presented here, obtained with single specimens of normal and HS blood, are representative of those obtained with blood samples from 5 hematologically normal persons and from each of 6 patients with hereditary spherocytosis; 3 of patients had been splenectomized.

It is noteworthy that in both normal and HS cells, adenosine causes a reversal of the inhibitory effect of fluoride and of iodoacetate (4), suggesting that certain products of adenosine metabolism are effective in removing the fluoride block.

Inhibition of orthophosphate-P^{32} exchange by fluoride implicates enolase as a point of blockage. This interpretation is supported by the enhancement of fluoride inhibition in the presence of additional unlabeled orthophosphate (final concentration, 10^{-3} M)(5). The finding that adenosine counteracts not only the effect of fluoride but also that of iodoacetate suggests that the effective products of purine riboside metabolism may be contributing directly to the formation of phosphoenol-pyruvate, perhaps by way of hydroxypyruvate, without prior conversion to triose phosphates.

It was deemed worthwhile to ascertain whether HS cells also differed from normal rbc with respect to the effect of fluoride on the rate of exchange of intracellular P^{32}-labeled phosphate with unlabeled extracellular phosphate. To this end blood from hematologically normal donors and from patients with HS was incubated with orthophosphate-P^{32} for 1 hour. The rbc were then separated by centrifugation and washed twice with fresh autogenous plasma. The labeled cells were finally mixed with fresh plasma in proportion to the original hematocrit and reincubated for 4 hours with or without added fluoride. Re-

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TABLE I. Comparison of Effect of Fluoride on Rate of Exchange of Intracellular P^{32}-Phosphate in Labeled Red Cells from Normal Donors and from Patients with Hereditary Spherocytosis (HS).

Hr of incubation	Normal cells			HS cells		
	Control,* No. additives	NaF added†	Control: NaF ratio	Control,* No. additives	NaF added†	Control: NaF ratio
	c.p.m./ml plasma‡			c.p.m./ml plasma‡		
1	8680	5120	1.69	4320	7780	.55
2	8460	6080	1.39	5720	9400	.60
3	8060	6380	1.26	6800	9780	.69
4	10340	7160	1.41	7900	10960	.72

These data, obtained with single specimens of normal and HS blood, are representative of those obtained with blood samples from 3 hematologically normal donors and from 3 previously splenectomized HS patients.

* P^{32}-labeled cells, incubated with fresh plasma.

† *Idem* and 5×10^{-3} M NaF (final concentration).

‡ c.p.m. = counts/min. obtained with a Geiger-Müller tube. The count was obtained from 0.05-ml aliquot of plasma which had been dried onto a small spot on filter paper.

sultant P^{32}-activity of the plasma was determined at hourly intervals (Table I). The data show that the addition of fluoride caused an increase in the rate of release of P^{32} from the labeled HS cells, whereas fluoride had the opposite effect on normal rbc. The data presented indicate that orthophosphate-P^{32} exchange in HS cells differs in a characteristic manner from that in normal human rbc. Although more information is needed for an adequate explanation of these findings, it seems likely that enzymes activated by Mg^{++}, such as enolase and ATPase, are involved. Whether the formation of calcium fluoride has any significant relation to the observation reported, possibly by bringing about changes in electrolyte distribution between rbc and plasma(6), cannot be stated at this time.

Summary. 1. The exchange of orthophosphate-P^{32} by normal human red blood cells (rbc) and rbc of patients with hereditary spherocytosis (HS) has been studied in the presence of NaF. 2. The flux of orthophosphate-P^{32} into HS cells could be inhibited by 10^{-3} M NaF whereas the flux into normal rbc remained unaffected by this concentration of fluoride. 3. The exchange of intracellular phosphate-P^{32} was studied similarly in normal P^{32}-labeled rbc and in P^{32}-labeled rbc from patients with HS. Addition of NaF caused an increase in the rate at which P^{32} was released from HS cells and caused a decrease in the rate of release of P^{32} from normal rbc.

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Effect of Fetal or Neonatal Immunization on Antibody Response of the Adult Rat.*† (22590)

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It has been reported that animals exposed to an antigen during prenatal or early postnatal life may fail to produce antibodies to the same antigen administered in later life. Traub(1) observed that in a mouse colony carrying the lymphocytic choriomeningitis virus, an intra-uterine infection resulted in offspring harboring the virus for many months without evidence of immunologic response. Owen(2) described the natural occurrence of reciprocal antigenic tolerance in genetically dissimilar dizygotic bovine twins having a common placental circulation and has more recently described experimental reproduction of this phenomenon in rats(3). Dunsford *et al.*(4) also described the existence of a blood group chimera in a human twin having circulating erythrocytes of both types O and A, though genotypically blood group O. Such observations are in accord with the theory of antibody production proposed by Burnet and Fenner(5), who introduced the concept of "self-markers," and postulated that at some time during prenatal or neonatal life, animals "take stock" of their own "antigens" and henceforth can recognize an antigen as either autogenous, by not reacting to it, or as foreign and responding with antibody production. This theory would imply that a foreign antigen introduced into an animal prior to this stage of self-recognition will thereafter be treated as autogenous, so that later exposure provokes no immunological response. Early attempts by these authors to provide an experimental basis for this concept were unsuccessful(6). Support for this hypothesis was given by Billingham, Brent and Medawar(7) who demonstrated that the breakdown of skin homografts between 2 incompatible strains of mice could be prevented or greatly postponed by injection of splenic or other living cells from the donor strain into recipient fetuses. Following this observation—termed "actively acquired tolerance"—numerous investigators attacked this problem with a variety of soluble and particulate antigens or their derivatives in several animal species. Some workers did not observe acquired tolerance following prenatal exposure to the antigen(6,8,9). Where tolerance was observed, relatively massive doses of antigen were injected into young animals over prolonged periods of time(8,10,11).

The present study was designed to parallel the work of Billingham, Brent and Medawar in attempting to induce actively acquired tolerance in rats through prenatal or neonatal exposure to a cellular antigen. However, our experiments differed from the British study in that: 1) Unlike the homologous species relationship in the latter study, an antigen from a zoologically distant species was used; 2) The antigen consisted of cells not capable of multiplication, *i.e.* sheep erythrocytes; 3) Immunological response was measured by an *in vitro* technic inherently capable of greater precision than observations on the survival of skin homografts. This approach was selected with the rationale that if tolerance resulting in prolonged skin homograft survival is based on depressed antibody response, such a depression should be demonstrable in a more clearly defined immune system susceptible to accurate antibody titration.

Materials and methods. Animals. Mature albino rats of the Wistar strain were used for breeding purposes. *Antigen.* Sheep blood, drawn aseptically from a single animal was preserved at 2 to 5°C in sterile modified Alsever's solution(12). It was considered advisable to use erythrocytes from a single

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TABLE I. Weights and Ages of Rats at Time of Experiment.

Group	No. of fetuses at laparotomy	No. of survivors at time of challenge	Age at time of challenge, wk	Wt, g Range	Wt, g Avg
A	20	5[*]	10	155–240	188
B	10	4	10	165–184	175
C	8	7	10	134–167	147
D	13	9	9	97–169	125

[*] 1 rat died during first bleeding. Difference in numbers between second and third columns is accounted for by perinatal mortality.

bleeding for both the primary exposure and subsequent challenge, so as to minimize untoward effects due to possible minor antigenic variations in erythrocytes taken from different sheep. The cells were washed 3 times with 0.85% sterile saline, and a 5% suspension in 0.85% saline was prepared for the injections. This suspension contained approximately 8.0 x 10^8 erythrocytes, per ml as determined by direct count. *Procedure for intrafetal injection.* Pregnant animals were anesthetized with ether and the peritoneum was entered through a low midline incision. The gravid uterus was gently exteriorized, one horn at a time, and kept moist with warm saline sponges. The fetuses could be clearly visualized through the distended, transparent uterine wall by oblique illumination. The RBC suspension was injected into the peritoneal cavity of each fetus by careful penetration through the uterus and the lateral abdominal wall of the fetus with a 0.25 cc tuberculin syringe and #27 hypodermic needle. After all fetuses in each uterine horn were injected, the uterus was carefully replaced in the abdomen, and the abdominal incision was closed in 2 layers with continuous #0000 chromic catgut sutures. *Determination of hemolysin titers.* Titer estimations were performed by means of a spectrophotometric procedure currently used in this laboratory. The titer is expressed as the dilution of hemolytic antibody required to lyse exactly 50.0% of a carefully standardized sheep RBC suspension in 15.0 minutes, in the presence of an excess of guinea pig complement. Determinations are generally reproducible to within 10% (13).

Experimental. Animals were mated by placing 2 to 4 females with 1 male for 3 to 5 days. It was thus possible to approximate the fetal age at the time of antigen injection. The pregnant animals were divided into 4 groups, and when the fetuses were about 15 days old (*i.e.*, 7 days prenatal), intrafetal injections were made as follows:

Group A—Intrafetal RBC: 0.03 cc of a 5% suspension of sheep RBC, injected into the peritoneal cavities of 15-day-old fetuses (7 days prenatal). *Group B—Saline controls*: 0.03 cc of 0.85% saline injected into the peritoneal cavities of 15-day-old fetuses. *Group C—Noninjected controls*: Pregnant animals were laparotomized but the fetuses were not injected. *Group D—Postnatal RBC*: 0.05 cc of a 5% suspension of sheep RBC injected intraperitoneally into newborn fetuses, within 24 hours after birth. The animals in Groups A and D received only a single inoculation of antigen. Those in Group A received approximately 2.4 x 10^7 erythrocytes while those in Group D received 4 x

TABLE II. Individual Rat Hemolysin Titers.[*]

Rat No.	6th day post-challenge titer	Rat No.	6th day post-challenge titer
A 1[†]	30	B 1	980
A 2	350	B 2	490
A 3	1260	B 3	1020
A 4	1450	B 4	1000
A 5	825		
C 1	655	D 1	1000
C 2	1850	D 2	490
C 3	1070	D 3	550
C 4	1130	D 4	1040
C 5	920	D 5	615
C 6	1620	D 6	290
C 7	610	D 7	220
		D 8	330
		D 9	800

[*] Pre-challenge baseline titers were less than 20 for all rat sera except A 4 which had a titer of 34.

[†] This rat was pregnant at time of challenge and was not included in statistical evaluation of results.

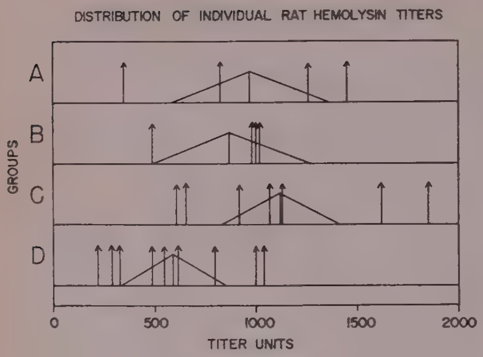

FIG. 1.

10^7 cells to compensate for increased body weight. The young were weaned at 20 to 25 days of age and maintained on the standard laboratory diet. At 9 or 10 weeks of age, each rat was bled from the ophthalmic venous plexus(14) to obtain 0.5 to 1.0 cc of blood for baseline determinations of hemolytic antibody. On the same day all animals were challenged with an intravenous injection of 1 cc of a 0.25% suspension of sheep RBC. On the 6th post-injection day, when anti-sheep RBC hemolysin titers are maximal(15), a second serum sample was obtained to determine the hemolytic antibody response.

Results. All animals were weighed at the time of weaning and at regular intervals thereafter until termination of the experiment. The growth rates in the 4 groups were entirely comparable. Final weights and ages of the rats at the time of challenge are shown in Table I. The baseline antisheep RBC hemolysin titers, determined in serum specimens drawn just prior to the challenge antigen injections, were less than 20 in all but one of the animals (see Table II). With respect to the post-challenge titers, there is no statistical difference between the intrafetal antigen injection group (A), the saline control group (B), and the non-injected rats (C). The titer of the postnatal antigen injection group (D) does not differ significantly from either A or B, although there is a significant difference (0.02 > P > 0.01) from non-injected rats (C) (Table III). Fig. 1 illustrates the distribution of individual rat hemolysin titers (arrows), as well as the mean titers (vertical lines) and 95% confidence limits of the mean (triangles).

TABLE III. Mean Titers on Sixth Post-Challenge Day.

Group	Mean titer	Stand. error of mean†	95% confidence limits of mean†
A	971 ± 490	192	587–1355
B	872 ± 254	192	488–1256
C	1122 ± 466	145	832–1412
D	591 ± 300	128	335– 847
	Pooled 383*		

* As there is no significant difference in stand. dev. of the 4 groups, the 4 values were pooled to obtain a more accurate stand. dev. based on a larger sample of animals.

† Calculated from this pooled stand. dev.

Discussion. These experiments demonstrate that rats exposed to a foreign antigen during the perinatal period retain the capacity for an unaltered antibody response to this antigen when mature. The findings are thus at variance with those of Billingham, Brent and Medawar, in that "actively acquired tolerance" was not observed. On the sixth post-challenge day, all animals but one exhibited a pronounced rise in hemolysin titer from baseline levels. The only rat which failed to respond proved to be pregnant during the challenge period. The mean post-challenge titers of the 4 groups, except those of groups C and D, are statistically similar. The mean titer of the postnatal RBC injected group (D) is significantly lower than that of the non-injected rats. Conceivably this difference may be due to the younger age of the group D animals, to their postnatal exposure to the amount of antigen injected, or to a combination of both factors. Even if the difference in titers is the specific result of neonatal exposure to the antigen, it seems unlikely that such a slight antibody depression might be expected to account for tolerance to homografts. The diminution of antibody response observed in the group D rats is far less pronounced than that reported by others in postnatally exposed animals(8,10,11).

In view of the disparity between the results of this and previous studies on immunologic unresponsiveness, an analysis of the differences in experimental conditions is indicated. As outlined above the perinatal exposure to antigen in the present work consisted of a single inoculation of a relatively small amount of cells incapable of multiplication. By contrast, tolerance was achieved in the previous studies when immature animals were subjected to a prolonged contact with relatively massive doses of antigen. In some instances the antigenic dosage was of sufficient intensity and duration so that prolonged persistence of circulating antigen was demonstrated(8,10). In other instances antigen administration consisted of daily injections for several months(11). A suppression of mean agglutinin titers was recently reported in groups of chickens exposed as embryos, to a single injection of dead *Salmonella pullorum* (16). Where individual titers were given by the author, the overlap in antibody response between the experimental and control chickens was essentially similar to that observed in the present work. Furthermore, the conclusions drawn in(16) were based on serum dilution titers and are not in accord with those derived from similar experiments in chickens performed by Cohn(9), who estimated the antibody response precisely in absolute weight units. In another recent study, also in accord with our findings, the intra-abdominal injection of 0.2 ml of rabbit blood into 6-day-old chick embryos, failed to suppress formation of natural heterohemagglutinins(17). Even tolerance conferred by living cells(7) may be a reflection of massive doses of antigen, in that surviving transplanted cells may continue to liberate antigen and thus saturate the recipient. This situation would obtain only if the cells are introduced into young animals prior to the appearance of the faculty of immunologic response. Moreover the animal's immaturity appears to be a facilitating rather than an essential condition, in that immunological capacity of the young is more easily overwhelmed than that of the mature animal. But even in the latter, massive antigen injection plus the superimposition of irradiation injury induces immunologic unresponsiveness to protein antigens(11). With pneumococcal polysaccharide, which persists in tissues for long periods(18), the administration of 5-50 μg to mice suffices to suppress antibody formation as shown in Felton's studies of "immune paralysis"(19). Indeed this author reports that there may be a quantitative relationship between the amount of pneumococcal polysaccharide required to induce immunologic paralysis and the specific immune state of the animal. Animals immunized when mature required a larger dose of antigen for the induction of immune paralysis. Finally, recent evidence indicates that even in "immune paralysis" antibody may be formed but is not detectable because of its interaction with antigen present in excess(20).

In retrospect therefore, is it not possible that this entire range of phenomena characterized by adversely altered immunologic response, although induced by various procedures, are not simply expressions of immune paralysis? Such an interpretation would emphasize a quantitative paralyzing basis to immunologic unresponsiveness rather than a delicate qualitative alteration in the antigen-host relationship, as would follow from the theory of Burnet and Fenner.

Summary. Rats exposed to sheep erythrocytes before or at time of birth exhibit an undiminished capacity for hemolysin production in response to a subsequent challenge with the same antigen given 9 to 10 weeks later. A possible explanation to account for the disparity between these results and those obtained by other investigators is offered.

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Sulfhydryl Factors in Degradation of Insulin-I^{131} by Liver Extracts.* (22591)

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The degradation of insulin-I^{131} by both a heat stable and a heat labile component of rat liver homogenate has been reported by Mirsky *et al.*(1). Lehmann and Schlossmann(2) had previously observed that insulin could be inactivated by a dialyzable heat stable factor in rabbit muscle extracts. This factor was able to reduce the disulfide linkages of insulin, and the authors concluded that it was a sulfhydryl compound such as glutathione (GSH) or cysteine. As liver has a relatively high content of GSH, it was of interest to find whether this compound could produce changes in the trichloroacetic acid (TCA) solubility of insulin-I^{131}. The present studies on the degradation of insulin-I^{131} in the presence of GSH were carried out in the hope of acquiring better understanding of the processes that might be involved in the inactivation of insulin by rat liver extracts.

Materials and methods. Crystalline zinc insulin, highly purified amorphous insulin, and crystalline glucagon were gifts of Eli Lilly and Co. The crystalline insulin was iodinated by Abbott Laboratories, and glucagon was kindly iodinated by Dr. Alfred Staub. Crystalline bovine plasma albumin and iodinated human serum albumin were obtained from the Armour Laboratories. Reagent grade GSH was a product of the Fisher Scientific Co., and oxidized glutathione (GSSG) was purchased from the Nutritional Biochemicals Corp. *p*-Chloromercuribenzoic acid (PCMB) was obtained from the Bios Labs, Inc. *Preparation of liver extracts.* Livers of decapitated 3-month-old Sprague-Dawley rats were perfused *in situ* with ice cold isotonic saline. The livers were then homogenized in 1.15% KCl with a glass homogenizer. The homogenate was diluted to a 10% tissue concentration and centrifuged for 25 minutes at 110,000 x *g*. The resultant supernatant fraction was used. For certain experiments, an acetone powder was prepared from the supernatant fraction. Prior to the assay procedure each 50 mg of powder was extracted with 1 ml of cold distilled water and material which had not dissolved at the end of 1 hour was removed by centrifugation. An aliquot of supernatant fraction from rat liver was placed in a boiling water bath for 10 minutes in order to prepare the heat stable factor. It was found in preliminary experiments that heating for 2 minutes was sufficient to produce maximum inactivation of the heat labile system which degraded insulin-I^{131}. *Measurement of insulin-I^{131} degradation.* 1 ml of liver extract or GSH solution that had been adjusted to pH 7.5 was preincubated for 5 minutes at 37°C in a 12 ml centrifuge tube. Then 1 ml of 0.067 M potassium phosphate buffer of pH 7.5, containing a trace quantity (0.1-2 μg) of dialyzed insulin-I^{131}, 1 mg of bovine plasma albumin,

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† Research Fellow, U.S.P.H.S.

and varying amounts of amorphous insulin carrier, was added. Each ml of substrate contained approximately 0.5 μc of radioactivity. The albumin was included in order to help stabilize the radioactive insulin(3). In control mixtures liver extract and GSH were omitted. All incubations were conducted in duplicate. At appropriate intervals, 1 ml of 20% TCA was added to terminate the reaction. The extent of degradation of insulin-I^{131} was measured as described previously(4). Duplicate determinations generally agreed within 5% of each other. It has been demonstrated that changes in the TCA solubility of insulin-I^{131} in the presence of liver extract are correlated with the formation of non-protein nitrogen from insulin and biological inactivation of the latter(4,5). *Studies with sulfhydryl reagents.* 1 ml of a 4% heated or unheated liver extract containing PCMB in a concentration of 6×10^{-4} M (an amount just sufficient to abolish the nitroprusside reaction for sulfhydryl groups) or GSSG in a concentration of 3×10^{-2} M was preincubated in a Dubnoff shaker for 30 minutes, shaking at a rate of 80 cycles per minute. 1 ml of phosphate buffer containing a trace quantity of insulin-I^{131} without carrier insulin was then added and incubation was continued for another 15 minutes, at which time the reaction was stopped with TCA. *Electrophoretic studies.* Four different incubation mixtures (A, B, C and D) were prepared in duplicate in 0.5 ml of 0.033 M phosphate buffer of pH 7.5 with the following components: (A) 20 μg (37 μc) of dialyzed insulin-I^{131}, (B) 20 μg of dialyzed insulin-I^{131} plus 0.25 mg of GSH (final concentration 1.6×10^{-3} M), (C) 10 μc of carrier-free NaI^{131}, and (D) 10 μc of NaI^{131}, plus 0.25 mg of GSH. Incubation was carried out in stoppered test tubes at 37°C for 1 hour. The tubes were then chilled in ice, and 0.3 ml of a 2% human plasma solution, 0.1 ml of 10% potassium iodide carrier solution, and 0.3 ml of 20% TCA were added rapidly with mixing. The mixtures were centrifuged, and 10 μl of each TCA supernatant were applied at the origin (O) of a strip of Whatman No. 1 filter paper moistened with 0.47 M potassium phosphate buffer of pH 7.7. The electrophoresis apparatus was that described by Grassmann and Hannig(6); the strips were run simultaneously in the cold room for 1 hour at a potential of 200 volts. The strips were then air-dried and counted with an end-window Geiger-Mueller tube arranged for automatic scanning and recording.‡ In a separate control experiment, 10 μc of carrier-free NaI^{131} were incubated with 20 μg of unlabeled insulin with or without the addition of 0.25 mg of GSH for 1 hour and then subjected to electrophoresis under conditions similar to those just described.

TABLE I. Effect of GSH upon Insulin-I^{131} in Presence of Varying Amounts of Amorphous Insulin Carrier.

Amorphous insulin, mg	Final GSH concentration, M	% TCA supernatant radioactivity in 20 min.
.02	8×10^{-3}	9.5
"	4 "	8.8
"	2 "	6.2
"	1 "	2.9
2.0	2×10^{-3}	1.3
.2	"	4.6
.02	"	6.0
.00	"	6.4

Results. Effect of GSH upon insulin-I^{131}. GSH was incubated in varying concentrations with a trace quantity of insulin-I^{131} and different amounts of amorphous insulin carrier. The results are shown in Table I. It is evident that glutathione produced degradation of the labeled insulin and that the amount of degradation tended to increase as the GSH concentration was raised, especially at lower levels of GSH. The percentage degradation of insulin-I^{131} fell only slightly with the addition of carrier insulin until relatively large amounts had been added.

In contrast to the behavior of iodinated insulin, radio-iodinated human serum albumin and glucagon-I^{131} showed no degradation when incubated with GSH in a concentration of 0.004 M for 30 minutes. This suggests that the effect of GSH upon insulin-I^{131} may not be one of direct deiodination of the intact protein molecule.

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‡ This instrument was kindly loaned by Dr. Donald J. Hanahan.

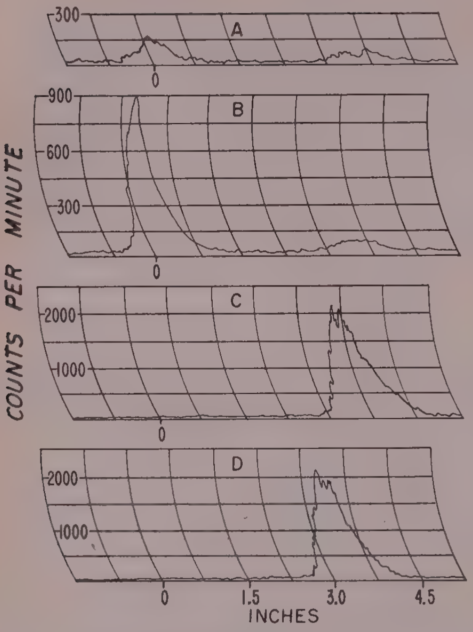

FIG. 1. Electrophoretic pattern of TCA soluble material resulting from action of GSH upon insulin-I^{131}.

Electrophoretic studies were conducted to test this possibility further. The results are shown in Fig. 1. Duplicate experiments yielded similar patterns. It is seen that incubation of insulin-I^{131} with GSH gave rise to a large amount of TCA soluble radioactivity, as shown in Strip B, which appeared as a band that migrated much more slowly than the iodide band of strip C. Strip D demonstrates that GSH itself does not produce such an alteration of the mobility of iodide. In a separate control experiment it was found that the presence of unlabeled crystalline zinc insulin, with or without the addition of GSH, similarly had no effect upon the migration of radioactive iodide. Therefore, the newly formed radioactive band near the origin probably represents an alteration of the insulin molecule rather than deiodination. Thus, the changes in TCA solubility of insulin-I^{131} produced by GSH may reflect reduction of the disulfide linkages of insulin, but activation of a proteolytic contaminant of the insulin cannot be ruled out.

Sulfhydryl nature of the heat stable and heat labile factors of liver. Mirsky and Broh-Kahn(7) and de Barbieri and Grassi (8) have found that liver homogenate treated with sulfhydryl inhibitors is less capable of inactivating insulin. It was of interest to see whether or not this inhibition extended to the heat stable component as well. PCMB and GSSG were chosen as reagents because both are among the more specific sulfhydryl inhibitors. The data of Table II show that PCMB in a concentration of 6 x 10^{-4} M completely inhibited the activity of boiled and unboiled liver extract. GSSG in a concentration of 3 x 10^{-2} was also able to inhibit the heat stable factor completely, but produced incomplete inhibition of the unheated extract. This suggests that the heat stable factor and heat labile factor both contain sulfhydryl groups which are essential for their activity, but that the sulfhydryl groups of the latter are less reactive towards GSSG. In control experiments the possibility that PCMB might cause precipitation of degradation products of insulin-I^{131} was ruled out.

As GSH could produce degradation of insulin-I^{131}, it was considered likely that the presence of this compound in liver extracts could account for the activity of the heat stable factor. However, it was found that when rat liver acetone powder extract was dialyzed against phosphate buffer of pH 6.2 for 48 hours and then heated in a boiling water bath for 10 minutes, it still retained considerable ability to degrade insulin-I^{131} (Table III). No GSH (TCA-soluble sulfhydryl material) could be detected in the dialyzed extract by the nitroprusside reaction under conditions which could detect GSH in a concentration of 5 x 10^{-5} M. Liver extract that had been precipitated and washed 3

TABLE II. Effect of Sulfhydryl Inhibitors upon Activity of Heat Stable and Heat Labile Factors of Liver.

Liver supernatant cell fraction	Inhibitor	TCA supernatant radioactivity, %
Unheated	None	73
	PCMB	0
	GSSG	39
Heated	None	6.2
	PCMB	0
	GSSG	0

TABLE III. Effect of Liver Acetone Powder Extracts Containing No Detectable GSH. .03 mg of carrier insulin added.

Liver preparation	Incubation, min.	TCA supernatant radioactivity, %
Dialyzed, unheated	10	10
	20	17
	30	23
	60	35
Dialyzed, heated	30	3.4
	60	6.7
TCA precipitated, heated	60	6.9

times with 5% TCA and then heated in a boiling water bath for 10 minutes was also able to degrade insulin-I^{131}. Thus, it is probable that protein-bound sulfhydryl groups contribute to the activity of the heat stable factor, but it is possible that some of these groups are less reactive in undenatured proteins.

Moreover, it was noted that GSH enhanced the degradation of insulin-I^{131} by liver extracts, and that this was more than a purely additive effect. This phenomenon (Table IV) would be compatible with an activation of a proteolytic enzyme system of liver by GSH. It is also possible that reduction of the insulin molecule may denature it and render it a better substrate for proteolytic enzyme action.

Reduction of the insulin molecule does not appear to be an absolute prerequisite for proteolytic degradation, however, because it was found that liver acetone powder extract after dialysis for 48 hours was still able to produce significant degradation of insulin-I^{131} (Table III). In this experiment 0.2 ml of dialyzed liver extract was incubated with insulin-I^{131} plus 0.03 mg of added carrier insulin. In another experiment, in which 0.3 ml of dialyzed extract was incubated with insulin-I^{131} in the absence of amorphous insulin, 73% of the radioactivity became TCA soluble in 10 minutes. Under conditions where insulin is completely inactivated(9), GSH was found to produce at most about a 25% increase in TCA supernatant radioactivity. Racker(10) has found that crude liver extracts can catalyze the reduction of insulin. Since the sites of cleavage of the insulin molecule are not known, and since GSH can affect the activity of the proteolytic enzyme system of liver which acts on insulin, it is difficult to distinguish the exact contributions of reductive and proteolytic processes when insulin-I^{131} is degraded by crude liver homogenates or by purified extracts in the presence of added GSH.

TABLE IV. Effect of GSH upon Degradation of Insulin-I^{131} by Liver Acetone Powder Extract. .03 mg of carrier insulin added.

Incubation time, min.	10	20	30	40	60
Reaction mixture additions	TCA supernatant radioactivity, %				
GSH	6	10	13	15	19
Liver extract	10	19	25	29	39
" " + GSH	16	30	43	53	69

Summary. 1. Glutathione degrades insulin-I^{131} and renders it more soluble in trichloroacetic acid. Evidence is presented that this is not the result of direct deiodination. 2. Rat liver extracts, even after exposure to a boiling water bath, can degrade insulin-I^{131}. This heat stable activity could be explained by the presence of sulfhydryl compounds in liver. 3. Reductive as well as proteolytic processes must be considered when analyzing the degradation of insulin by crude liver extracts.

The authors are indebted to Miss Ruth Miller for valuable technical assistance.

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Effect of Proteolytic Enzymes on Granuloma Formation. (22592)

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Recent studies have demonstrated the potent anti-inflammatory anti-edema properties of proteolytic enzymes(1-3). These studies have utilized protection against edema formation or erythema as a measure of the activity of the enzymes. Recently Ungar and Ginsburg reported a marked depression of granulation tissue surrounding surgically implanted gut in the livers of trypsin treated rats(4). Sherry and co-workers(5) also reported that they were able to prevent post-operative adhesions in dogs treated with plasminogen activators and attribute these results to activation of the blood fibrinolytic system. The purpose of this report is to present data demonstrating the effectiveness of the proteolytic enzymes, trypsin and chymotrypsin, as agents capable of modifying the tissue response to a foreign body *viz.* implanted cotton pellets.

Material and methods. Sprague-Dawley female rats weighing 95-110 g were used. The technic of Stucki and Meyer(6) was used for subcutaneous implantation of the weighed cotton pellets. The enzymes were subcutaneously administered as an aqueous solution daily, beginning immediately after pellet implantation and continued for 5 days. On the 6th day the animals were sacrificed, the pellets with the surrounding granulation tissue removed and dried at 105°C for 18 hours. The dried granuloma pellets were weighed, the pellet weight subtracted from the entire dry weight and the remainder recorded as granuloma weight. Control animals were injected with 0.5 ml 10% gelatin solution. One series of animals was treated with heat inactivated trypsin. *Pellet implantation* sites which had become infected were discarded. This situation occurred in several instances in the control animals, but was not observed in the enzyme treated rats. Korth and Bock(7) have similarly recorded a reduced incidence of infectivity in trypsin treated mice. The *enzymes* used were 2 x crystallized trypsin and chymotrypsin prepared by the method of Kunitz *et al.*(12).

TABLE I.

No. animals	Treatment	Dose, mg	Granuloma wt, mg	% inhibition
6	Controls		15.4 ± .93	
7	Trypsin	5	10.3 ± .98	33
7	Controls		13.7 ±1.80	
7	Chymotrypsin	2.5	6.8 ±1.37	51
14	Controls		8.8 ± .64	
14	Trypsin	2.5	6.0 ± .34	32
14	Chymotrypsin	2.5	6.0 ± .42	30
14	Controls		8.1 ±1.30	
14	Trypsin	1	5.5 ± .38	32
14	Chymotrypsin	1	7.2 ± .42	10
6	Controls		8.4 ± .83	
6	Trypsin (Heat inactivated)	5	9.0 ± .91	
	Hypophysectomized animals			
10	Control		8.5 ±1.31	
6	Trypsin	2.5	5.2 ± .58	39
6	Chymotrypsin	2.5	5.1 ± .67	40

Results. The data obtained are presented in Table I. The decrease in granuloma formation was consistently obtained in each of the 5 individual experiments. A quantitative effect could not be significantly demonstrated over a 2-fold increase in dose of enzyme. When the dose is reduced to 1 mg it is noted that although trypsin was effective in reducing the weight of the granuloma, chymotrypsin was ineffective. This difference may be due to the relative proteolytic activity of the 2 enzymes. Martin(8) has noted a correlation between effectiveness of the proteolytic enzyme as an inhibitor of egg white edema and proteolytic activity. Heat inactivated trypsin was ineffective in decreasing the size of granulomas.

Discussion. The effect of the proteolytic enzymes, trypsin and chymotrypsin, upon the granuloma induced by subcutaneous implantation of cotton pellets is another manifestation of the anti-inflammatory properties of these substances. The mode of action is not clear but presumably may be dissociated from an activation of the plasma fibrinolytic system, inasmuch as chymotrypsin, which is active in reducing the granuloma reaction, is ineffective as an activator of the plasma fibrinolysin system(9,10). Since the effect upon granuloma size occurs in hypophysectomized animals (Table I) this action cannot be ascribed to a stimulation of the pituitary-adrenal system with consequent hypersecretion of cortical steroids. This indicates that the enzymes probably exert their action either through their own proteolytic or depolymerase properties, or by the activation of an anti-inflammatory mechanism distinct from the profibrinolysin-fibrinolysin system. Martin(11) has suggested that these enzymes may be adsorbed or concentrated at the site of fibrin deposits and may exert their anti-inflammatory properties by depolymerization or digestion of these fibrin deposits.

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During the course of these experiments it was also observed that the incision area of the trypsin and chymotrypsin treated animals healed with less granulation tissue than did the similar areas in the untreated control group.

Summary. The parenteral administration of trypsin and chymotrypsin to animals carrying subcutaneous implants of cotton pellets, significantly reduces the size of the granulomas formed around these pellets. Heat inactivated enzyme does not manifest this property.

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Effect of Antimetabolites on Growth of *Endamoeba histolytica*. II. Pantetheine and Coenzyme A Antagonist.* (22593)

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The identity of a number of growth factors for *Endamoeba histolytica* has recently been established by Nakamura and Baker (1,2). Purine and pyrimidine analogues were found to be potent inhibitors of the amebas by Nakamura and Jonsson(3). Aside from this report and that of Kun *et al.*(4), who studied the effect of metabolic inhibitors on carbon dioxide and hydrogen sulfide production by *E. histolytica,* no other information is available with regard to the effect of antimetabolites on the amebas. This situation arises from the fact that until recently very little was known regarding the specific nutritional requirements of this protozoan. Since coenzyme A has been identified as a growth factor for *E. histolytica*(2), it became desirable to confirm this observation using a coenzyme A antagonist.

Boxer *et al.*(5) reported that β-*d*-pantoyl-

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aminoethanethiol (PAET) was an antagonist of the pantetheine-coenzyme A system *in vitro*. PAET inhibited the synthesis of coenzyme A from pantetheine and also the activity of coenzyme A itself. Structurally PAET contains all the functional groups of pantetheine but in a different molecular spacing.

The present studies have shown that the disulfide of PAET, bis (N-pantoyl-β-aminoethyl) disulfide (PAED),[†] is a potent inhibitor of *E. histolytica*.

Materials and methods. All experiments were performed with the NRS strain of *E. histolytica* under bacteria-free conditions employing technics described earlier(1-3). Briefly, the basal assay medium consisted of egg slants overlaid with serum-Ringer solution, dextrose, penicillin G, streptomycin, rice powder and sodium thioglycollate. The antagonist, PAED, was studied in the basal medium and also in the basal medium fortified with the growth factors adenosinetriphosphate (ATP) and ribose-5-phosphate (R-5-P). Calcium pantothenate and coenzyme A (coA) used for reversal studies were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and pantetheine was obtained from the California Foundation for Biochemical Research, Los Angeles, Calif.

Results. PAED inhibited *E. histolytica* under bacteria-free conditions in dilutions of 1/1,000 to 1/50,000 (1 mg/ml to 0.02 mg/ml). This inhibition, as shown in Table I, was completely reversed by pantetheine and partially reversed by coA. Calcium pantothenate was inactive. PAED prevented growth of amebas even in media fortified with the growth factors ATP and R-5-P. In cultures of amebas containing associated bacteria (stock cultures), PAED was totally inactive due perhaps to an adequate supply of pantetheine or coA being continuously produced by the bacteria.

TABLE I. Inhibition of Bacteria-Free Cultures of *E. histolytica* by PAED and Reversal of Inhibition by Pantetheine and coA.

Material assayed	Total No. of determinations	Avg count of amebas per low power field at end of 3 days
Basal (control)	40	1
Basal + ATP + R-5-P	36	133
Basal + calcium pantothenate (1 mg/ml)	10	5
Basal + pantetheine (1 mg/ml)	9	64
Basal + coA (1 mg/ml)	7	41
Basal + PAED (.1 mg/ml) + ATP + R-5-P	4	0
Basal + PAED (.02 mg/ml) + ATP + R-5-P	4	0
Basal + PAED (.01 mg/ml) + ATP + R-5-P	4	11
Basal + PAED (.02 mg/ml) + ATP + R-5-P + calcium pantothenate (1 mg/ml)	4	0
Basal + PAED (.02 mg/ml) + ATP + R-5-P + pantetheine (.1 mg/ml)	6	101
Basal + PAED (.02 mg/ml) + ATP + R-5-P + coA (.5 mg/ml)	6	70
Basal + PAED (.02 mg/ml) + pantetheine (.1 mg/ml)	4	62
Basal + PAED (.02 mg/ml) + coA (.5 mg/ml)	4	37
Stock cultures (bacteria present)	70	51
Stock cultures + PAED (1 mg/ml)	4	53

Discussion. Since both pantetheine and coA have been reported as growth-stimulatory substances for amebas(2), it is understandable that PAED was a potent inhibitor of *E. histolytica*. The reversal of inhibition by pantetheine and coA suggests strongly that PAED is an antimetabolite and not a non-specific chemical toxic agent; however, these data do not allow us to determine whether this inhibition is competitive or non-competitive. Boxer *et al.*(5) have suggested possible mechanisms of action of the antagonist: (1) the antagonist prevents synthesis of coA from pantetheine, and (2) that an analogue of coA, containing the PAED moiety instead of pantetheine, is enzymatically synthesized. In amebic metabolism it is probable that the antagonist functions by blocking conversion of pantothenic acid to pantetheine, although it is also possible that it acts by preventing the conversion of pantetheine to coA. Although Boxer *et al.*(5) found some inhibition of coA itself by PAET, we have found that supplementation of the media with coA allowed the amebas to overcome inhibition by PAED. This establishes the importance of coA in the nutrition of *E. histolytica.*

[†] Obtained through the courtesy of Dr. Andrew N. Wilson, Chemical Division, Merck and Co., Rahway, N. J.

Summary. 1. PAED, a pantetheine antagonist, inhibited bacteria-free cultures of *E. histolytica.* 2. Reversal of inhibition was possible with pantetheine and coenzyme A, but not with pantothenic acid. 3. The possible mechanism of action of PAED is discussed.

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The Cyclic Disulfide Ring of Oxytocin.* (22594)

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The structure of oxytocin, the principal oxytocic hormone of the posterior pituitary gland, has been established through its synthesis(1,2) as the cyclic disulfide of L-cysteinyl - L-tyrosyl - L-isoleucyl - L-glutaminyl-L-asparaginyl - L-cysteinyl - L-prolyl - L-leucylglycinamide, which is represented by I. On the basis of structural studies, it has been proposed that the antidiuretic hormone from oxen is structurally similar to oxytocin(3,4), with L-phenylalanine and L-arginine in vasopressin replacing the L-isoleucine and L-leucine, respectively, of oxytocin. An octapeptide amide synthesized according to this structure has been found qualitatively to have the expected biological activity(5). Moreover, vasopressin isolated from the hog has been found to differ in composition from beef vasopressin by the substitution of lysine for arginine(6). It has been suggested that both these vasopressins are otherwise structurally identical(3), and this has received strong support from the results of recent studies on the synthesis of lysine vasopressin(7).

CyS-Tyr-Ileu-Glu(NH_2)-Asp(NH_2)-CyS-Pro-
Leu-Gly(NH_2)
I

Although some of the more well established physiological activities of oxytocin, namely, the oxytocic, milk-ejecting, and avian vasodepressor effects, are strikingly different from the pressor and antidiuretic activities of vasopressin, it may be noted that qualitatively these hormones possess several biological activities in common. Thus the vasopressins also have some oxytocic, avian vasodepressor(8,9) and milk-ejecting activity(9-11), and oxytocin also possesses low but definite degrees of pressor and antidiuretic activity(12), in addition to the other physiological activities of each of these hormones.

A striking structural feature of both of these hormones is a 20-membered cystine-containing peptide ring. It is of interest that in the structure of insulin as postulated by Sanger and his associates, an intrachain disulfide ring of the same size as that which occurs in oxytocin and vasopressin has also been proposed(13).

One interesting speculation resulting from these observations is that the ring structure itself of the posterior pituitary hormones might possess intrinsic biological activity. Ac-

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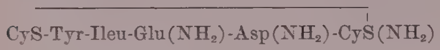
tivity inherent in the ring might then account for the presence of many of the same physiological activities in oxytocin and vasopressin. In this connection and as part of a general study in this laboratory of the relationship of structure to the biological activity of the posterior pituitary hormones, the synthesis of the compound, the cyclic disulfide of L-cysteinyl - L-tyrosyl - L-isoleucyl - L-glutaminyl-L-asparaginyl - L-cysteinamide, represented by II, was undertaken.


$\overline{\text{CyS-Tyr-Ileu-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS}}\text{(NH}_2\text{)}$

II

This compound represents the ring moiety of oxytocin, where the prolylleucylglycinamide side chain linked to the cyclic portion of oxytocin has been replaced simply by an amide grouping. The C-terminal residue of the resulting peptide is therefore a half-cystine amide residue which forms part of the 20-membered ring, being linked through its amino group to the carboxyl group of the asparagine residue and through its sulfur to the other half-cystine residue which bears the free amino group.

Of the several routes possible for preparing this compound the one described was chosen because of its similarity to the route used originally in the synthesis of oxytocin(1,2). By the use of a parallel approach, it has been possible to take advantage of the fact that several intermediates common to both these compounds could be used. Thus the tripeptide and dipeptide derivatives, tosyl-L-isoleucyl - L-glutaminyl - L-asparagine(14) and carbobenzoxy-S-benzyl - L-cysteinyl - L-tyrosine(15), respectively, which had been used previously in the synthetic oxytocin work also served as intermediates in the synthesis of the cyclic disulfide II. However, for the tetrapeptide amide S-benzyl - L-cysteinyl - L-prolyl - L-leucylglycinamide(16) used in the former synthesis, the amino acid derivative S-benzyl - L-cysteinamide has now been substituted. The yields in this synthesis, however, were somewhat lower.

Experimental.† S-benzyl - L-cysteinamide was prepared by the amination of ethyl S-benzyl-L-cysteinate‡ in methanolic ammonia. A suspension of 7.9 g of ethyl S-benzyl-L-cysteinate hydrochloride in 50 cc of ethyl acetate was treated with 7.9 cc of triethylamine for 30 minutes. It was then filtered and concentrated and the residue was dissolved in 135 cc of anhydrous methanol saturated with ammonia. After 3 days the solvent and NH_3 were removed and the residue was crystallized from ethyl acetate, 5.5 g (92%); m.p. 78.5-79.5°. A sample of the free base was recrystallized from toluene; m.p. 80°, $[\alpha]_D^{23} + 5.65°$ (c 3, acetic acid).

Anal. Calcd. for $C_{10}H_{14}ON_2S$: S, 15.3; N, 13.3. Found: S, 15.2; N, 13.3.

The S-benzyl - L-cysteinamide (0.56 g) was coupled with tosyl-L-isoleucyl - L-glutaminyl-L-asparagine(1.0 g) at 95° for 1 hour in 10 cc of diethyl phosphite by the use of 1.4 cc of tetraethyl pyrophosphite(17). The protected tetrapeptide tosyl-L-isoleucyl-L-glutaminyl - L-asparaginyl-S-benzyl - L-cysteinamide was precipitated with water and washed with warm methanol; wt. 1.0 g, m.p. 249-250°. After several crystallizations from acetic acid or diethyl phosphite a sample melted at 273-274°.

Anal. Calcd. for $C_{32}H_{45}O_8N_7S_2$: C, 53.4; H, 6.3; S, 8.9. Found: C, 53.7; H, 6.3; S, 8.8. Removal of the S-benzyl and N-tosyl protecting groups and rebenzylation was effected in 70% yield by treatment of 100 mg of the protected tetrapeptide with sodium in liquid ammonia followed by 0.05 cc of benzyl chloride in 0.5 cc of toluene(18,19). The isoleucyl-glutaminyl-asparaginyl - S - benzylcysteinamide‡‡ obtained was purified by countercurrent distribution(20) in the system 2-butanol-0.01 M NH_3 in which the partition coefficient was approximately 1.0. A suspension of 100 mg of this tetrapeptide in 0.5 cc of diethyl phosphite containing 0.1 cc of tetraethyl pyrophosphite was allowed to react with 135 mg of N-carbobenzoxy S-benzyl-L-cysteinyl - L-tyrosine at 85° for one-half hour. The resulting protected hexapeptide N-carbobenzoxy-S-benzyl - L-cysteinyl - L-tyrosyl - L-isoleucyl - L-glutaminyl - L-asparaginyl-S-benzyl-L-cysteinamide, which was also prepared by the use of the coupling agent N, N′-dicyclohexylcarbodiimide(21), was washed with warm methanol; wt. 93 mg. When it was reprecipitated from formamide it melted at 249-250°. This was treated with sodium in liquid ammonia to remove the protecting groups(18) and after evaporation of the ammonia the sulfhydryl product was aerated in aqueous solution at pH 6.5(1,2), after which the solution was adjusted to pH 3.5, concentrated under reduced pressure and finally dried by lyophilization. The material was isolated by the use of countercurrent distribution in the system 0.05% acetic acid-2-butanol. The distribution of material was determined by following the absorption in the ultraviolet at 275 mμ and the distribution of weight. The contents of a peak having the partition coefficient $K = 0.19$ were concentrated and lyophilized and yielded a white powder, $[\alpha]_D^{23} - 20.5°$ (c 0.51, water).

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† The reported melting points are corrected, capillary melting points.

‡ This compound was prepared according to Harington, C. R., and Pitt Rivers, R. V.(15). It melted at 161-162°; reported, 156-157°.

‡‡ When the tetrapeptide was used without purification, several byproducts were isolated along with the final product. Studies on these products will be reported separately.

For analysis the compound was dried at 100°C *in vacuo* and showed a loss in weight of 3.9%. The analytical values are calculated on the basis of the dried sample and are corrected for 1.0% ash.

Anal. Calc'd. for $C_{30}H_{45}O_9N_9S_2 \cdot C_2H_4O_2$: C, 48.1; H, 6.2; N, 15.8. Found: C, 47.8; H, 6.5; N, 16.0. When the material was hydrolyzed in acid and analyzed on the starch column according to the quantitative procedure of Moore and Stein(22), it showed the expected amino acid composition in the following ratio: isoleucine 1.0, tyrosine 0.8, glutamic acid 1.0, aspartic acid 1.1, cystine 1.0, ammonia 3.6. Like oxytocin, this polypeptide underwent cleavage into two peptides on treatment with bromine water(2).

Results. The purified peptide was assayed for the principal physiological activities normally associated with oxytocin, namely, the oxytocic, milk-ejecting, and avian vasodepressor activities, the latter being the effect on the blood pressure of the fowl. The USP Standard posterior lobe powder served as the reference in each of these determinations. The cyclic peptide was found to have a low but definite degree of oxytocic activity. When measured on the isolated rat uterus according to the method of Burn(23[a,b]), it assayed approximately 3.3 U/mg. It was therefore roughly 1/150 as active as oxytocin and approximately 1/10 as active as beef vasopressin in this respect. The cyclic peptide was also found to be active in effecting the letdown of milk,[§] the potency being approximately 1.1 U/mg. These activities, while low, are considered real because of the great sensitivity of the assay methods. The sensitivity of the uterus method is approximately 2 mU, and in the assay for milk-ejecting activity, 0.5 mU can be detected(12). The material was also assayed for strepogenin activity by the method used previously for related peptides(24) and had a potency of 55 U/mg.[‖]

The most striking aspect of the pharmacology of the cyclic peptide was concerned with the avian vasodepressor effect. The depressor effect of oxytocin on the blood pressure of the chicken has been associated with its oxytocic activity(25) and in pure oxytocin the former activity is present to the same degree as the oxytocic activity. The official assay for the hormone adopted by the United States Pharmacopeia(26) is in fact based on the avian blood pressure depressor method. Assay of the cyclic peptide, however, for this effect on blood pressure by the method of Coon(27) using the anesthetized chicken showed that, although the compound possessed significant oxytocic and milk-ejecting activities, the avian depressor effect was absent within the limits of the assay method. Any avian depressor activity, if present, would therefore be less than approximately 0.03 U/mg. The small pressor effect which oxytocin has been found to possess(12) was also not detectable in the cyclic peptide.

[§] The assays for milk-ejecting activity were kindly performed by Dr. H. B. van Dyke, Mr. S. L. Engel, and Dr. K. Adamsons, Jr. of the College of Physicians and Surgeons, Columbia University, to whom appreciation is expressed. The assay procedure involved measurements of the pressure within the lactating mammary gland of the rabbit.

[‖] Strepogenin activity was determined through the kindness of Dr. D. W. Woolley and Dr. R. B. Merrifield of the Rockefeller Institute for Medical Research.

Considerable interest arises from a comparison of the ratio of the potencies of the biological effects of the peptide with that ratio in oxytocin. Thus, while the oxytocic, milk-ejecting, and avian depressor activities of the hormone are equipotent in terms of the standard, the corresponding ratio of these activities in the cyclic peptide is approximately $3:1:<0.03$. There is, therefore, 3 times as much oxytocic as milk-ejecting activity per unit of weight of the peptide. Simplification of the structure of oxytocin has thus resulted in a markedly disproportionate change in the potencies relative to each other of the several physiological activities of the hormone. It is conceivable that this type of change may be a general consequence of structural alteration when dealing with compounds that exhibit multiple physiological activities.

The virtual absence of avian depressor activity in the cyclic peptide and the difference in the relative oxytocic and milk-ejecting activities afford a pharmacological differentiation of it from the hormone. The compound has been differentiated from oxytocin in another way, *i.e.*, by means of a comparison of the degree of potentiation of the oxytocic effect brought about by the presence of magnesium ion. This *in vitro* effect of magnesium has been described by Fraser(28) in experiments in which the oxytocic activity of various preparations of oxytocin and vasopressin of low potency as well as of mixtures of these was determined using the guinea pig uterus in a medium containing graded amounts of magnesium. The cyclic peptide was assayed directly against a solution of pure synthetic oxytocin diluted to approximately the same potency in modified Locke solution(23^b) using the rat uterus. A similar assay was performed in Tyrode solution (29), which contained 10 mg% $MgCl_2$. The oxytocic activity of the cyclic peptide was increased approximately 10 times in this concentration of magnesium ion, while the synthetic oxytocin underwent approximately 63 fold potentiation.

It could be suspected that the relatively low degree of biological activity in this peptide may not be a property of the compound itself, but may be due possibly to the presence of a small amount of oxytocin picked up as a contaminant in the laboratory. However, the absence of avian depressor activity in the cyclic pentapeptide, the differences between oxytocin and the cyclic peptide with respect to the ratio of the oxytocic/milk-ejecting potencies and the degree of potentiation of the oxytocic effect by magnesium ion, as well as the fact that the final product was extensively purified, make this possibility extremely unlikely.

The results of the bioassays of the peptide allow certain deductions with respect to some of the structural requirements for biological activity in oxytocin and closely related compounds. The significant degree of activity possessed by the peptide that represents the cyclic moiety of oxytocin makes it clear that the entire molecule of oxytocin is not necessary for the existence of milk-ejecting and oxytocic activity. The fact that the activity of the cyclic peptide is of a relatively low order of magnitude could suggest that the minimum requirements for milk-ejecting and oxytocic activities in oxytocin might possibly be defined by the cyclic part of the oxytocin molecule. The tripeptide side chain of oxytocin or portions of the side chain would then serve to enhance these biological activities. For significant avian depressor activity to be present in these peptides, however, there would appear to be different, perhaps more extensive structural requirements. It may be added in this connection that several other peptides closely related in structure to oxytocin have been tested and have been found not to have significant activity. Thus, the linear peptide the disulfide of L-isoleucyl-L-glutaminyl - L-asparaginyl - L-cysteinyl - L-prolyl - L-leucylglycinamide(24) which represents an even larger, although partly different, portion of the sequence of the amino acids of oxytocin than does the cyclic peptide has been found to possess negligible oxytocic, milk-ejecting,§ and avian depressor activity. Preliminary testing of an aqueous solution of the disulfide of L-isoleucyl - L-glutaminyl-L-asparaginyl - L-cysteinamide obtained by the treatment of the corresponding tosyl S-benzyl peptide with sodium in liquid ammonia followed by aeration without further purification showed that this compound also seems to have negligible oxytocic and avian depressor activity.

Comparison of the highly active oxytocin, the weakly active cyclic pentapeptide, and the inactive heptapeptide and tetrapeptide disulfides with respect to their potencies of oxytocic, milk-ejecting, and avian depressor activity show clearly that a considerable degree of biological specificity is involved here. However, inasmuch as oxytocin and the two vasopressins do share these activities to varying degrees and inasmuch as the cyclic peptide possesses two of them, it is apparent that certain structural changes are allowable. It would seem that perhaps within a necessary framework of the molecule, a minimum requirement of which may be the cyclic part, some change, such as the substitution of certain of the amino acids by others, may be tolerated without complete destruction of these three biological activities of oxytocin.

It is of interest that there appears to be a completely different type of structural specificity involved in the biological effect associated with strepogenin activity. Thus, the otherwise inactive heptapeptide disulfide was found to be almost as active as oxytocin itself in stimulating the growth of *L. casei*(24). On the other hand the cyclic pentapeptide which does have significant oxytocic and milk-ejecting activities also has considerable strepogenin activity, and this activity is less than the strepogenin activity of the heptapeptide disulfide. It has been indicated that chain length of the peptide may be of some importance in determining the strepogenin activity for *L. casei*(30), and the strepogenin activity of the cyclic peptide would not be inconsistent with such a viewpoint.

Summary. A cyclic pentapeptide amide, the disulfide of L-cysteinyl - L-tyrosyl - L-isoleucyl - L-glutaminyl - L-asparaginyl - L-cysteinamide, which represents the cyclic moiety of oxytocin has been prepared synthetically and some of its physical and chemical properties are given. This compound has been found to have low but significant oxytocic (3.3 U/mg) and milk-ejecting (1.1 U/mg) activity but no detectable avian depressor activity. Moreover, this simplification of the oxytocin molecule appears to have resulted in a change in the ratio of the potencies of oxytocic, milk-ejecting, and avian depressor effects, the ratio being $3:1:<0.03$ for the cyclic pentapeptide amide compared to 1:1:1 for oxytocin. Some of the possible structural requirements for biological activity in oxytocin have been discussed in the light of these results.

The author wishes to thank Mr. Joseph Albert for carrying out the microanalyses, Dr. Paula Zimmering for the starch chromatography, Miss Lorraine Silverman for her assistance, and Mrs. Sylvia Kirsimagi White for the oxytocic and blood pressure assays reported herein. The stimulating discussions of the problem with Dr. Vincent du Vigneaud are acknowledged with pleasure.

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Eight Culture Strains (Detroit) of Human Epithelial-Like Cells.*† (22595)

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Various investigators have reported the development of stable strains of human cells capable of growing on glass surfaces in relatively simple fluid media(1-8). Some strains have been derived from tumor tissue and others from presumably normal tissue. Most have been described as "epithelial" although there are also human fibroblast-like strains in existence. Virologists have given a great deal of attention to one of these human cell strains, the HeLa strain(1), and wide use of this strain has stimulated the development of other human cell strains which might have additional or different useful properties.

A total of 8 epithelial-like strains have now been isolated in these laboratories(9,10). The present report is concerned with histories of the cell strains, procedures used for their isolation and maintenance, and preliminary observations on their morphology and growth behavior.

Histories of the cell strains. Five strains (Detroit-6, -32, -34, -52 and -98) were derived from cultures of sternal bone marrow. Two (Detroit-30A and -56A) were cultured from carcinomatous ascitic fluids and one (Detroit-116P) was obtained from lymphomatous pleural fluid. Table I lists the origin of each cell strain, pertinent data on the

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TABLE I. Histories of Detroit Strains of Epithelial-Like Cells.

Cell strain	Tissue source	Diagnosis of patient's disease	Biopsy material containing malignant cells	Appearance of plaques (days)	Life of strain (mo)*
Detroit- 6	Sternal marrow	Carcinoma of lung	Mass in lung	51	19
” - 32	” ”	Carcinomatosis, primary site undetermined	Pleural fluid	65	13
” - 34	” ”	Metastatic carcinoma of bone	Tumor of femur	51	13
” - 30A	Ascitic fluid	Carcinoma of breast	Mass in breast, ascitic fluid	24	16
” - 56A	” ”	Carcinomatosis, primary site undetermined	Ascitic fluid	55	10
” -116P	Pleural ”	Lymphosarcoma	Pleural ”	21	8
” - 52	Sternal marrow	Diabetes Mellitus†	None	57	10
” - 98	” ”	Refractive error†	”	53	7

* To June 1, 1956. † No malignancies detected.

human donor, the age of the primary culture when epithelial-like cells were first detected, and the subsequent interval (to the present time) during which each strain has been maintained.

Methods of isolation and maintenance. Heparinized aspirated marrow specimens were centrifuged at 4-10°C for 15 minutes at 40 g. The supernatant plasma containing suspended marrow cells was diluted with nutrient solution so that the final mixture contained 125,000 or more cells per ml. Eight ml were placed into each of a series of 3-ounce prescription bottles.‡ The size of the cell inoculum did not appear to be a critical factor between the ranges of 125,000 and 250,000 cells per ml.

Ascitic and pleural fluids were diluted 1:1 in nutritive medium and cultured in 8 ml aliquots without further manipulation. Varying admixture with erythrocytes did not appear to have any deleterious effects. In all instances the bottles were incubated on their flat sides at 36°C.

The nutrient medium used originally was composed of 40% human cord serum, 2% chicken embryo extract, and 58% Hanks' balanced salt solution with phenol red in a final concentration of 0.002% as a pH indicator ($CS_{40}BSS_{58}EE_2$). The nutrient medium in primary cultures was replaced when the pH fell below a range of 6.8 to 7.0. Daily observations were made through the flat sides of the bottles with an ordinary light microscope at 60X or 100X magnification. At intervals, the supernatant fluid containing unattached cells was removed, then centrifuged for 10 minutes at 750 g. Smears of the cell sediments were stained with May-Grünwald-Giemsa for morphologic study. The appearances of such cells in primary marrow cultures have been described in detail(2).

Observations on primary cultures. The marrow cultures from which epithelial-like cells were isolated passed through 3 phases. The first phase was characterized by the presence of specific myeloid cells among which mitotic figures were present. These cells gradually declined in number. The second phase was that of the appearance of monocytoid cells, reticulum cells and macrophages which could be recognized in the culture bottles as large discrete round cells firmly attached to glass. This was followed by the third phase in which spindle-shaped fibroblast-like cells became visible. These phases usually overlapped one another and were variable in time of onset and duration but the sequence of phases was consistent. The carcinomatous ascitic fluids and lymphomatous pleural fluid differed from the marrows in that the fibroblast-like cells appeared within a few days together with the large round cells of monocytoid and macrophage types.

Development of epithelial-like cells. In each instance, the epithelial-like cells were first recognized as isolated plaques or colonies

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‡ Owens Oval 3-oz prescription bottles of Duraglas, obtained from McKesson & Robbins, Inc., Detroit.

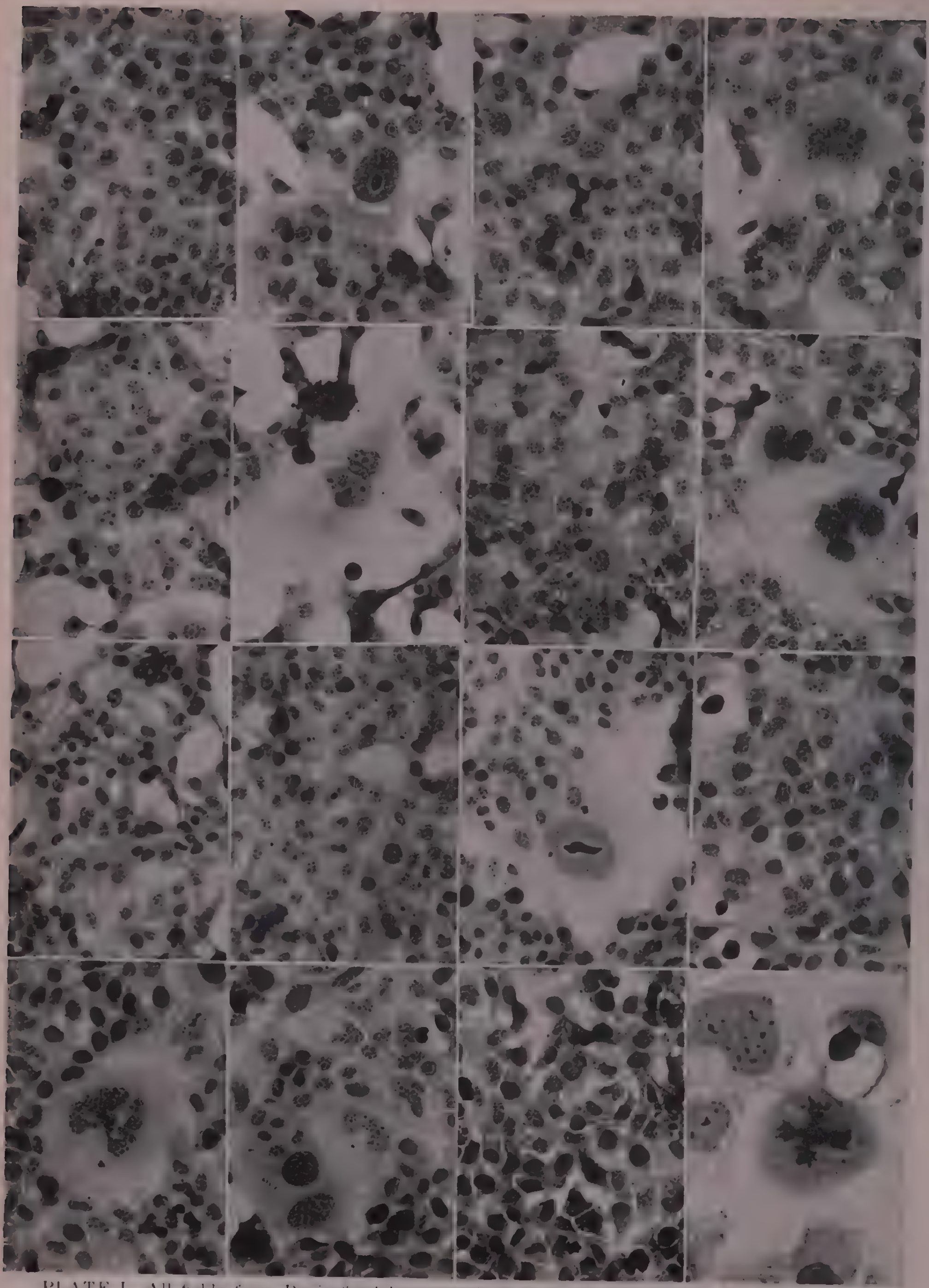

PLATE I. All fields from Bouin fixed hematoxylin-stained coverslip preparations. Magnifications are 180× except the field in the lower right hand corner, which is 450×. *Top row* (left to right): 1. Detroit-6. 2. Polyploid cell in Detroit-6 culture. 3. Detroit-30A. 4. Polyploid

cell in Detroit-30A culture. *Second row* (left to right): 1. Detroit-32. 2. Polyploid cell in Detroit-32 culture. 3. Detroit-34. 4. Polyploid cells in Detroit-34 culture. *Third row* (left to right): 1. Detroit-52. 2. Detroit-56A. 3. Polyploid cell in Detroit-56A culture. 4. Detroit-98. *Bottom row* (left to right): 1. Polyploid cell in Detroit-98 culture. 2. Polyploid cells in Detroit-98 culture. 3. Detroit-116P. 4. Multipolar mitosis in Detroit-116P culture.

of polygonal cells sometime after the appearance of a fibroblastic phase of growth. Following the development of the isolated plaques, cords and groups of polygonal cells extended from them to form confluent sheets on the glass surface. In several instances, the plaques or portions of the sheets of cells of the primary cultures were removed mechanically for preparing subcultures that continued to grow as sheets of polygonal cells. Subsequently, it was found that frequent subculture after freeing attached cells by trypsin solution resulted in survival of polygonal cells from mixed populations of such cells and spindle-shaped elements. Once the epithelial-like polygonal cells were established in subcultures, there were no reversions to a fibroblast-like form.

Subculture and maintenance of the epithelial-like strains. Originally the cell strains were subcultured and maintained in CS_{40}-EE_2BSS_{58}. In this medium, however, the cultures occasionally were observed to go through irregular periods of poor growth. Following the reports of Eagle(11), all strains have been maintained in Basal Medium (Eagle)¶ plus 20% human cord serum ($BME_{80}CS_{20}$), streptomycin 50 μg/ml and penicillin 50 units/ml. Subculturing is accomplished by replacement of the medium with 5 ml of 0.25% trypsin solution (Difco 1:250) in BME for 10 minutes at 37°C. The trypsin-treated cell suspensions are centrifuged, resuspended in $BME_{80}CS_{20}$ prepared with Hanks' balanced salt solution rather than Earle's as recommended by Eagle(11). The latter modification was introduced in order to maintain the medium at a pH of 7.2 to 7.4 rather than 7.8 to 8.0. This minimizes clumping of cells and results in improved attachment of cells to the surfaces of the culture bottles. Approximately 50,000 cells/ml are placed in the 3-oz prescription bottles in aliquots of 8 ml and incubated at 36°C. Subsequent feedings between transfers or subcultures are made with $BME_{80}CS_{20}$ prepared with Earle's solution. Within 24 hours, sheets of cells are evident. After 3 days confluent sheets cover the flat surfaces of the bottles and after 4 to 5 days, with only one intervening feeding, all the strains yield averages of 400,000 to 500,000 cells/ml.

Preliminary observations on differences and similarities of strains. Plate I illustrates stained cells from sheets of tissue grown on coverslips inserted into Beckman beakers(1) or Leighton tubes.‖ All of the 8 strains grow in the fashion of epithelium, especially as squamous epithelium in the sense these terms are used in the tissue culture literature(12, 13). Furthermore, the 8 Detroit strains cannot be distinguished from each other on the basis of examination of individual cells by ordinary methods of microscopy applied to living or fixed stained preparations. Each of the strains exhibits features found in malignant tissues, namely, anisocytosis, giant nucleoli, polyploidy, abnormal mitoses, and rapid growth even though some of them were derived from tissues of patients free of malignant diseases. On the other hand, there are subtle differences among the various strains. These are evident in the topographic appearances of the sheets of cells that form during proliferation. In Plate II are photomicrographs made from living cultures in the prescription bottles after 3 days of growth in $BME_{90}CS_{10}$. At this stage of growth Detroit-98 and -116P are similar; they appear as confluent monocellular layers. After the same period of incubation, Detroit-52 grows as clumps of rounded cells. Continuous epithelial-like sheets do not appear until the fourth or fifth day of growth. The other cell strains cannot be distinguished by their topographic

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¶ Obtained as separate stock concentrates of amino acids, vitamins and glutamine from Microbiological Associates, Inc., Bethesda, Md.

‖ Obtained from Microbiological Associates, Inc., Bethesda, Md.

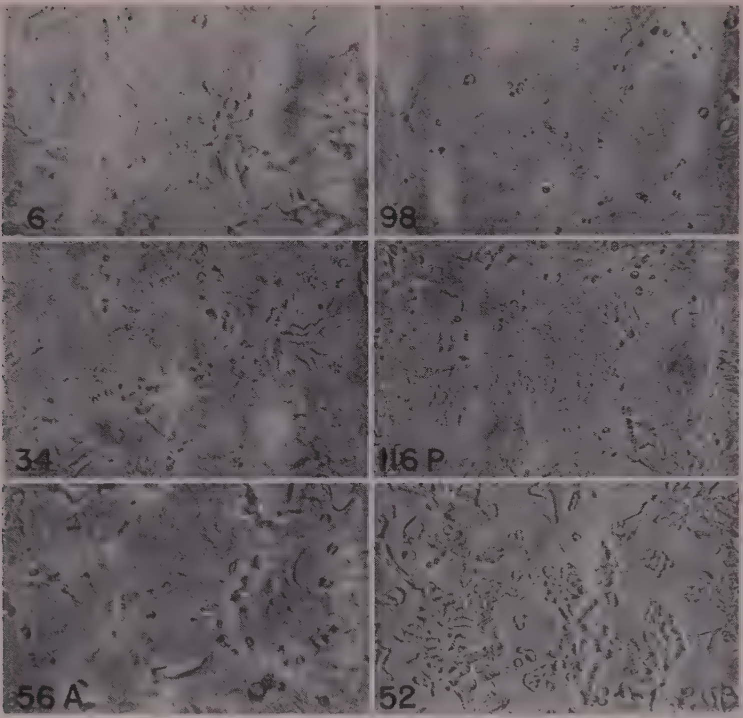

PLATE II. Appearances of unstained living cultures as seen through the flat surfaces of prescription bottles after 3 days of growth. (75×.)

appearances under these conditions (10% serum). If the serum content of the medium is increased to 20%, all strains form confluent monocellular layers earlier.

Discussion. It is emphasized that the designation of the 8 Detroit strains discussed herein as epithelial-like does not imply that they are necessarily true epithelial cells, nor do cytologic phenomena of the types seen in malignant tissues imply that such morphologic features, by themselves, indicate that the cells are cancerous. The cells reported here have growth characteristics that are similar to those of cells derived from epithelial tissues. In the absence of any signs of morphologic differentiation toward the formation of tubules or adenomatous structures these cells must be regarded as examples of "indifferent epithelial-like cells" mentioned by Parker(14). The 8 Detroit strains reported here have derived from tissues free of epithelium as well as from sources known to contain malignant epithelial cells. In each instance, however, the primary material contained a mixture of cells including derivatives of the histiocytic or reticuloendothelial system and the vascular system as well as hemic elements. Although various cell strains developed by others have been referred to as lung cancer cells, skin cells, cervical cancer cells, bone marrow cells, etc., there exists the possibility that the various strains may have developed from a source common to the different tissues used, for example, vascular endothelial cells or histiocytes. Distinctions between morphologically similar strains of cells must be based on more precise morphologic technics and biological activities than have usually been used. An attempt to make use of comparative virological susceptibilities as a means of distinguishing between the various epithelial-like strains will be reported in another communication. It is now apparent that the development of strains of human epithelial-like cells from bone marrow, ascitic and pleural fluids, can be accomplished by fairly simple methods but the explanation of how and why such cells appear in cultures is more difficult. For example, it was stated by Parker *et al.*(15) that they had observed numerous instances of the development of strains of unidentified cells that multiplied with great rapidity to yield uniform populations of free-living cells. It was implied that such cells were altered in some way during cultivation *in vitro*. The idea is similar to that expressed in discussions of cell modulation(16,17). The environmental conditions that account for the appearances of altered cells or modulation forms are not completely understood. It has been demonstrated by numerous investigators that morphologic and growth characteristics of cells can be modified greatly by deficiencies of essential nutrients. Eagle(11) has recently demonstrated the structural changes resulting from some amino acid, vitamin and salt deficiencies. Morphologic studies have emphasized the need for such information. Leighton *et al.*(6) have described the transformation of normal human fibroblasts into cells that appeared malignant by histologic criteria. It is questionable whether histologic features alone are adequate for determining the malignant potentialities of cells. Some of the morphologic criteria of malignancy are found in Detroit strains that were derived from noncancerous individuals. The possibility that such strains have become malignant *in vitro* requires proof by methods that have not yet been devised for study of human strains. Puck *et al.*(18) described the isolation of clones of cells from HeLa cultures. The clones represent different phenotypes derived from the original population of cells. The ideas of Parker *et al.*, Leighton *et al.*, and Puck *et al.*, are equally reasonable explanations of the appearance of epithelial-like cells in tissue cultures derived from mixed populations of cells. An unresolved problem relates to the possibility that the stable strains of epithelial-like cells represent the survival of a particular cell type included in the primary cultures.

Summary. The histories of 8 strains of human epithelial-like cells derived from bone marrow, ascitic and pleural fluids have been described. Preliminary observations on their morphology have been reported and the significance of these observations has been discussed.

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Dietary Protein and Serum Cholesterol in Rats. (22596)

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Undernutrition has been shown to enhance atherosclerosis and hypercholesterolemia in cholesterol-fed chickens(1) and rabbits(2). The role played by protein intake in this phenomenon is not quite clear. Diets high in casein(3) or defatted beef(4) have been reported to be atherogenic for rabbits, although vegetable protein appeared to have no such effect(5). Recently, cholesterol-fed rabbits were maintained on low calorie, high protein diets. and while atherosclerosis was not reduced in these animals, some lowering of the levels of serum cholesterol was observed(6). The purpose of the present investigation was to study the effect of varying amounts of dietary protein on the serum cholesterol of rats being fed this sterol.

Method. Two experiments were carried out: in the first, casein was the protein fed to groups of rats, in the second, Drackett protein* was used (Table I). The diets in the casein series (A to E) contained 10, 10, 20, 40 and 60% protein as noted. No choline chloride was present in diet A, while 0.5% of the compound was added to the remainder of the casein diets. In the Drackett protein series (K to M) the percentages of the protein were 10, 40 and 60, supplemented in each case by 2% choline chloride. Table I gives the percentages of diet components. Protein was increased at the expense of sucrose, and cholic acid(7) was added in order to heighten cholesterolemia. All diets contained 25% lard, 5% H.M.W. salt mix(8), 2% cholesterol, 1% cholic acid and the following vitamin mixture (mg/kg): thiamin 10, riboflavin 10, pyridoxine 10, niacin 10, Ca pantothenate 30, inositol 100, p-aminobenzoic acid 10, folic acid 1, biotin 0.1, mixed tocopherols 40, menadione 1; plus Vit. A 7200 u/kg and Vit. D 1200 u/kg. Diets A-E also contained 4% alphacel. Rats were males of the Charles River strain. Those in Exp. 1 weighed 180 g initially; in Exp. 2, 200 g. After 3 weeks (Exp. 1) or 4 weeks (Exp. 2) on the various diets, all animals were exsanguinated. The livers were pooled by groups and dissolved in 20% KOH for subsequent analysis, and the sera were assayed individually for total cholesterol, using the method of Trinder(9).

Results. Results are given in the Table. The average serum cholesterol value in Group B was 5 times higher than in Group A; while the percentage of liver cholesterol was almost half.

The effects of the incorporation of different percentages of casein in diets are apparent from an examination of data on Groups B through E. When casein comprised 10% of the diet, the average serum cholesterol value was 1132; at 20% it was 658; and at 40%, 460. The average weight of the rats also increased with greater protein intake, as would be expected. However, 60% casein did not produce a further lowering of serum cholesterol and weight gain as compared with 40% casein.

In Exp. 2, where Drackett protein was fed, similar results were obtained in animals receiving increasing amounts of dietary protein. The average serum cholesterol level dropped in Groups K, L and M, the weight of rats rose, and liver weight increased as percentages of protein were raised. In contrast to the observation made in Exp. 1, 60% protein continued to influence these values beyond those at 40%. Whether the higher percentage of choline in the Drackett diets may be related to this difference is uncertain.

The differences between the serum cholesterol level of groups A and B, A and D, C and B and L and M are highly significant ($p < 0.001$) and those between groups C and D and L and K are significant ($p < 0.05$ and $p < 0.03$, respectively).

Summary. Increasing the protein content

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* Purified soybean protein prepared by the Drackett Products Co., Cincinnati, O.

TABLE I. Autopsy Data* on Rats Fed Various Levels of Dietary Protein.

Group	Diet components (%)			Wt gain (g)	Serum chol. (mg %)	Liver wt (g)	Liver lipid distribution	
	Protein	CCL†	Sucrose				% NS‡	% chol.
Exp. 1								
A	10 C§	—	53.0	− 4	228 (121- 299)	11.8	19.4	9.3
B	10 C	0.5	52.5	−14	1132 (885-1500)	10.5	12.3	5.0
C	20 C	0.5	42.5	96	658 (423- 945)	16.7	13.3	5.7
D	40 C	0.5	22.5	113	460 (274- 983)	16.4	16.9	5.3
E	60 C	0.5	2.5	72	483 (351- 728)	15.2	10.8	5.0
Exp. 2								
K	10 D‖	2	55	−10	637 (341- 978)	12.6	—	—
L	40 D	2	25	174	382 (198- 520)	16.4	—	—
M	60 D	2	5	164	188 (141- 258)	17.2	—	—

* Averages for groups of 6 rats each. † CCL—Choline chloride. ‡ NS—Nonsaponifiable. § Casein. ‖ D—Drackett protein.

of the diets of groups of rats on a normally hypercholesterolemic regimen (cholesterol plus cholic acid) caused a progressive drop in serum cholesterol values. This observation held true when either casein or Drackett protein was used. All diets except one contained added choline chloride. Animals on the choline-free diet had lower serum cholesterol levels and higher liver cholesterol levels than a comparable choline-fed group.

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Serum Glycoproteins in Thyroidectomized Rats. (22597)

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Robbins, Petermann and Rall(1) found that the thyroxine-binding protein of human serum has a solubility, sedimentation constant and electrophoretic mobility in alkaline solution resembling an α-2-glycoprotein. Gersh (2) pointed out that thyroid cells present submicroscopic particles containing glycoproteins and that thyroid colloid has at least 2 proteins with carbohydrate-reacting groups. One of these proteins seems to be identical with thyroglobulin.

However, we were unable to find any reference in the literature dealing with serum glycoproteins in thyroidectomized animals. This paper reports the results of our experiments on the effect of thyroidectomy in serum glycoproteins.

Materials and methods. Male Wistar rats weighing between 50 and 80 g were thyroidectomized under ethyl urethane anesthesia. These animals and a group of normal Wistar rats of the same age, sex and weight were given the normal laboratory diet. After 45 days all animals were bled by heart puncture under ether anesthesia. Blood was left to clot and serum separated. Serum glycoproteins

TABLE I. Glycoproteins and Total Proteins in Sera of Normal and Thyroidectomized Rats.*

Group	No. of rats	Glycoproteins, mg hexose/100 ml	Total proteins, g/100 ml	G/P, mg glycoprotein/g protein
		Mean ± stand. dev.†		
Normal	12	125 ± 9	7.71 ± .53	16 ± 1.35
Thyroidectomized	8	98 ± 12	6.41 ± .76	15 ± 2.00

* Mean body wt 161 g.

† Stand. dev. $= \sqrt{\dfrac{\Sigma (x-\bar{x})^2}{n-1}}$.

were determined as total protein-bound hexose using a galactose-mannose standard according to Winzler's technic(3). The determination of total serum proteins was carried out by the biuret method of Weichselbaum (4).

Results. Average values for serum glycoproteins and total proteins obtained with normal and thyroidectomized rats, followed by their standard deviations are given in Table I. Glycoproteins and total proteins were expressed as mg of galactose-mannose and g of protein per 100 ml of serum, respectively. The G/P ratio was expressed in mg of glycoprotein per g of total protein.

The averages obtained were all highly significant ($P<0.001$) according to Student's t test(5). Glycoprotein levels in thyroidectomized rats were markedly lower than those obtained for normal ones. The difference was highly significant ($t = 5.5$, $P<0.001$). Total proteins were also considerably lowered in thyroidectomized animals, and the difference between the averages for operated and normal rats was also statistically significant ($t = 4.5$, $P<0.001$). On the other hand, G/P ratios of normal and thyroidectomized rats were practically the same and the difference between the averages of both groups was not statistically significant ($t = 1.335$, $P<0.2$).

Discussion. Our studies in thyroidectomized rats are in accordance with those of Podhradszky(6,7) who reported a decrease on total serum proteins in thyroidectomized dogs. In view of our results the observed decrease in serum glycoproteins after thyroidectomy was probably a consequence of hypoproteinemia, as it was shown by similar values of G/P ratio observed in both normal and operated rats.

Summary. Glycoproteins and total proteins were determined in sera of normal and thyroidectomized male Wistar rats. Statistically significant decreases of serum glycoproteins and total proteins were observed in the operated animals. Our results seemed to show that the fall in serum glycoproteins of thyroidectomized rats is related to the hypoproteinemia observed in this experimental condition.

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Propagation of Fibroma Virus in Tissue Cultures of Cottontail Testes.

(22598)

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This paper describes propagation of Shope fibroma virus through 14 successive passages in cultures of cottontail (*Sylvilagus* sp.) testes and through a few passages in similar cultures of testes from domestic rabbits. References to propagation of fully active fibroma virus *in vitro* have not been encountered(1).

Materials and methods. Virus. Fibroma virus employed was one originally isolated from a wild cottontail(2). Two lines of this virus were propagated *in vitro*. One line, designated A, had been passed intradermally in cottontails, and another, B, was the same virus after 2 intravenous passages, also in cottontails. Propagation in tissue cultures was initiated with 10% suspensions of skin fibromas. *Tissue cultures.* Although some mature cottontail testes were used in later passages, testes from immature or from non-breeding animals were selected by preference. Testes of domestic rabbits were uniformly selected from immature animals. The tissues were minced with scissors prior to fixation in roller tubes by means of chicken plasma clot. Cultures were fed maintenance solution(3) plus 20% horse serum initially and 10% horse serum in 4 to 8 days when cellular growth was well started and the cultures were inoculated. Each tube contained 2 ml of supernatant fluid. At intervals of 3 to 10 days (usually 4 or 5) transfers were made by the inoculation of tubes with 0.2 ml of fluid from the previous passage. Monolayer monkey kidney cells(4) and explants of human foreskin were cultivated under similar conditions. *Tests for virus.* Presence and amounts of virus in supernatant fluids were determined by intradermal inoculation of domestic rabbits by methods previously described(2). Maintenance fluid containing 10% horse serum was used as a diluent in titrations and in storage of virus. *Animals.* Albino rabbits were procured from local dealers, and cottontails were purchased from dealers in Wyoming and Kansas or were trapped locally. *Histology.* For histological examinations testis tissue grown on cover slips was fixed in Bouin's solution and stained with hematoxylin and eosin.

Results. Cottontail testes cultures. The 14 passages through which lines A and B of fibroma virus were carried represented 18 and 20 tenfold dilutions respectively of the original fibroma suspensions. Since these original suspensions titrated only 10^{-5}, a considerable increase of virus occurred. Multiplication of fibroma virus was evident within individual passages. Fig. 1 demonstrates such results of skin tests in a domestic rabbit. Third passage of line A, with virus barely demonstrable in undiluted supernatant fluids obtained 3 days after inoculation, had a virus titer of 10^{-2} at 7 days and of 10^{-3} at 13 days after inoculations. In sixth passage, there was a rise of from 10^{-3} at 13 days to 10^{-4} between 4 and 8 days after inoculation, fluids having been changed and titrated at each interval. In all passages, with either line of virus, 10^{-4} was the maximum virus titer obtained. Titers usually declined at later intervals when the tissue culture cells were degenerating. Such degeneration, after a variable number of weeks, was equally demonstrable in uninoculated control cultures.

Cultures of domestic rabbit testes. The only success in propagation of fibroma virus in cultures of testes from domestic rabbits was with line A virus, which was recoverable, although barely so, from fourth passage supernatant fluid. There was suggestive evidence of virus multiplication in the first 2 passages. Thus, in the second passage, supernatant fluid had a virus titer of 10^{-2} 4 days after inocula-

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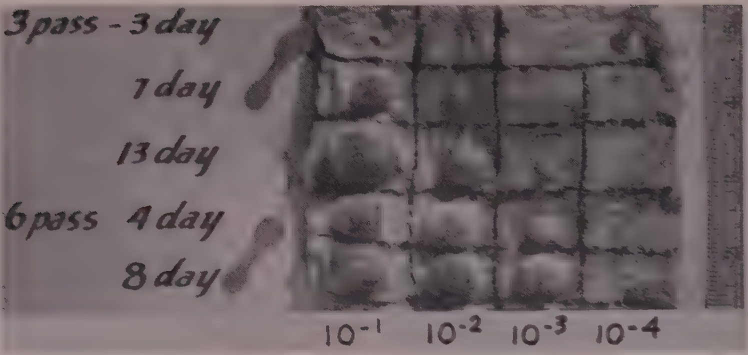
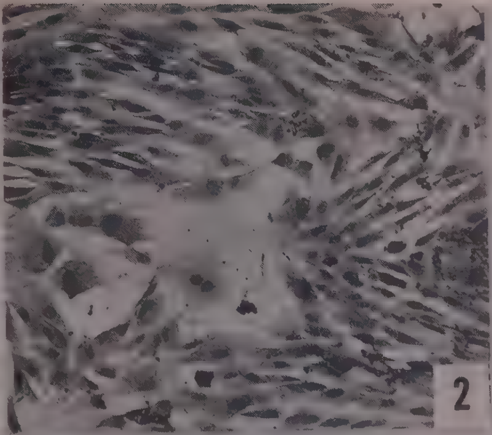


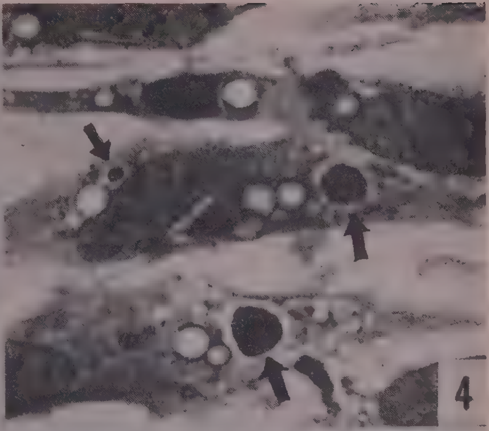
FIG. 1. Skin of a domestic rabbit, showing results of titrations of fibroma virus as it occurred in supernatant fluids of various tissue culture passages. Photograph taken 8 days after skin inoculations were made.

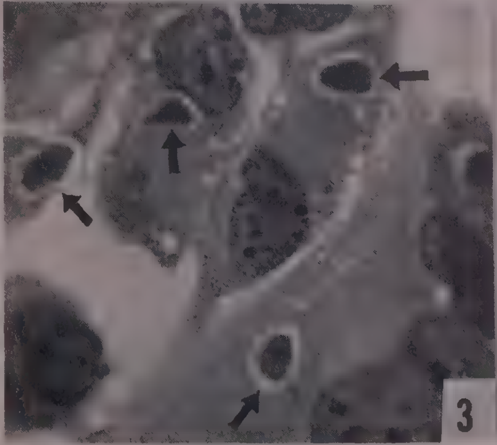
tion. After 2 complete changes of fluid in the course of 6 days, the supernatant fluid, of the same passage and 10 days after inoculation, had a titer of 10^{-3}. This was the same titer obtained by titrations of fluid from the first passage 4 and 8 days after inoculation. Other attempts at propagation of fibroma virus were unsuccessful. Line B virus from the original suspension and viruses from the third passage of lines A and B in cottontail testis cultures survived only a single passage in cultures of testes from domestic rabbits.

Negative results with other types of tissue. Virus multiplication did not occur in other types of tissue culture inoculated with original virus suspension of lines A and B. Both lines of virus were recoverable from the first but not from a second passage in human foreskin. Line B of fibroma virus was twice inoculated into cultures of monkey kidney cells. In both attempts the virus barely survived to a third passage, which represented only 3 tenfold dilutions of the starting suspensions.

Histology. A predominance of fibroblasts, surrounding islands of epithelial cells, characterized tissue cultures of both cottontail and domestic rabbit testes. Epithelial cells were more prevalent in the latter cultures. Fig. 2 illustrates proliferation of fibroblasts, as seen in an infected culture of cottontail testis. Uninoculated tissue cultures had a similar general appearance, for there was no evidence of cell destruction resulting from infection. Acidophilic, intracytoplasmic inclusions, irregular in size and shape and surrounded by a clear zone or halo, were abundant on first passage of virus in tissue cultures, whether of rabbit or of cottontail origin (Fig. 3). Occasionally, a single fibroblast might contain 2 inclusions. Inclusion bodies were less abundant on subsequent passage, but when present, they were concentrated in groups of closely adjacent cells. Similar cells in surrounding areas appeared normal. Fig. 4 represents a culture of fibroma virus (7th tissue culture passage) in cottontail testis tissue which had been inoculated 3 weeks previously. In this preparation inclusion bodies are associated with intracytoplasmic vacuoles. These vacuoles had a ground-glass appearance, apparently due to mucin-like contents. In common with the associated inclusions, they increased in prominence between the first (Fig. 2) and third weeks following inoculation. The vacuoles were encountered primarily in areas where the inclusions were also present and they were not observed in control, uninoculated cultures. Fig. 5 shows a section of a testis fibroma from an infected cottontail. It shows elongate vacuoles, some filled with a similar mucin-like substance, as well as 2 acidophilic, intracytoplasmic inclusions. Clear vacuoles, often in clusters and nearly filling the cytoplasm of certain cells, were occasionally found in both normal and infected tissue cultures. They were readily distinguishable from the vacuoles having a ground glass appearance and illustrated in Fig. 4. Using the periodic acid-Schiff (PAS) method, Fisher(5,6) described intracytoplas-







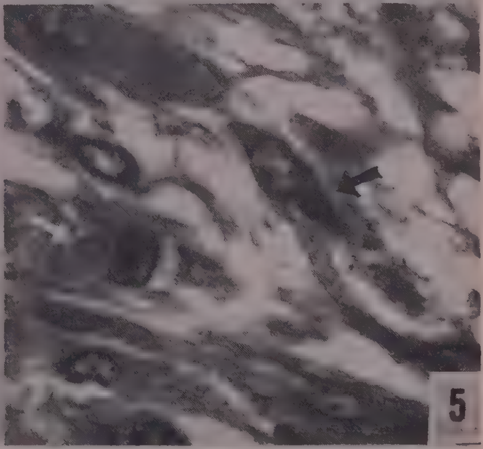


FIG. 2. Growth of fibroblasts from cottontail testis inoculated with 7th passage fibroma virus 5 days previously. (H & E 96×.)

FIG. 3. Acidophilic, intracytoplasmic inclusions (arrows) in tissue culture of cottontail testis, inoculated with fibroma virus 4 days previously. (H & E 1000×.)

FIG. 4. Intracytoplasmic inclusions (arrows) and associated vacuoles containing mucin-like substance. Seventh passage of fibroma virus, 3 wk after inoculation into tissue culture of cottontail testis. (H & E 1000×.)

FIG. 5. Section of fibroma which developed in testis of cottontail following inoculation. Inclusion bodies shown by arrows. (H & E 1000×.)

mic inclusions within fibroma cells of the corium which could not be stained with hematoxylin and eosin. He believed that these inclusions were possibly glycoprotein in nature and not related to the virus particles. Although PAS-positive inclusions were found in testis fibromas, they were not nearly as abundant as in fibroma cells of the corium in skin tumors of the same cottontail. So far I have had no definite evidence of the occurrence of this type of inclusion body in tissue culture preparations.

Discussion. Evidence is presented that fibroma virus multiplies in tissue cultures of cottontail testes. Two features of testicular fibromas, such as develop in cottontails following inoculation, are apparent in stained preparations of tissue cultures. They are acidophilic inclusions and secondly vacuoles filled with a mucin-like substance. The inclusions closely resemble those described by Feller, Enders, and Weller(7) in their study of vaccinia virus in roller tube cultures of chick fibroblasts. This resemblance is possibly another expression of the relation of pox viruses to those of fibroma and myxoma(8). Propagation of fibroma virus in tissue culture offers a means of studying the nature of this tumor-inducing virus, its effect on living cells, and its transformation into strains of greater or lesser virulence.

Summary. (1) A mammalian tumor-inducing virus, that of the Shope fibroma, has been propagated in tissue culture. (2) Two lines of fibroma virus were each carried through 14 successive passages in tissue cultures of cottontail testes. (3) One line of virus was carried up to 4 passages in similar cultures of domestic rabbit testes. (4) Growth of virus was accompanied by formation of acidophilic, intracytoplasmic inclusions and by vacuoles containing a substance with a ground glass appearance.

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Effect of Diethylstilbestrol on Plasma 17-hydroxycorticosteroid Levels in Humans.* (22599)

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Evidence to indicate a stimulatory effect of estrogens on the pituitary-adrenocortical system in animals has been reviewed by Roberts and Szego(1). In the guinea pig, Zondek and Burstein(2) found increased urinary corticosteroid excretion following estrogen administration. On the other hand, Vogt(3) has measured directly adrenocortical hormones in the adrenal venous blood of the rat and rabbit, and found diminution of secretion following administration of hexoestrol in the rat but not in the rabbit. In the human, Gemzell(4) found elevated plasma 17-hydroxycorticosteroids during pregnancy, and suggested that such elevations are due to adrenal activation from the increased estrogen which occurs during pregnancy.

This study is concerned with plasma 17-hydroxycorticosteroid levels following diethylstilbestrol (stilboestrol) administration to human subjects, and was carried out because of the finding of markedly elevated levels in a patient with carcinoma of the prostate while on stilboestrol therapy.

Procedure. Plasma 17-hydroxycorticosteroids were determined by the method of Nelson and Samuels(5), blood being drawn between 8:30 and 9:30 a. m. In this laboratory levels in 30 normal adults of both sexes were 14.4 ± 6.2 (mean ± S.D.) μg per 100 ml plasma (range 3.3 to 28.4); these values are similar to those of Bliss *et al.*(6).

Seven men with carcinoma of the prostate, one male (E.W.) with Addison's disease of 12 years duration, and one female (F.P.) who had undergone subtotal adrenalectomy for Cushing's syndrome were studied. In the patients with carcinoma of the prostate, pre-

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TABLE I. 17-Hydroxycorticosteroid Plasma Levels ($\mu g/100$ ml) in 7 Patients with Carcinoma of Prostate Treated with Stilboestrol.

Last previous stilboestrol	Before				Stilboestrol, 20 mg/day, on days 9-18				After		
	Day 1	4	8	Mean	11	15	18	Mean	22	25	29
14 days	21.4	15.5	20.5	(19.1)	30.8	33.4	14.7	(26.3)	27.4	26.3	29.4
2 years	14.7	10.1	7.5	(10.8)	33.0	14.0	30.5	(25.8)	30.4	15.2	13.4
34 days	11.4	11.7	9.9	(11.0)	54.3	44.5	47.5	(48.8)	36.7	20.2	17.9
8 "	23.7	28.3	25.6	(25.9)	36.1	41.2	34.4	(37.2)	35.6	21.6	22.3
7 "	29.4	19.8	—	(24.6)	—	41.9	39.6	(40.8)	41.5	26.4	22.8
7 "	22.4	22.4	29.4	(24.7)	40.2	30.2	34.8	(35.1)	13.4	20.0	13.4
30 "	22.9	22.0	25.1	(23.3)	48.6	39.8	62.5	(50.3)	—	—	—
			Mean	(19.9)			Mean	(37.8)			

vious treatment with stilboestrol was discontinued, and after an interval without this drug, 17-hydroxycorticosteroid blood levels were determined before, during, and after a 10-day course of stilboestrol, 20 mg per day orally in 2 doses. The same dose was given to the 2 other patients. For comparative purposes and at a later period the patient with subtotal adrenalectomy was tested with ACTH, using 25 i.u. in an intravenous infusion over a 6-hour period on 2 successive days, with blood samples taken at the beginning and end of the infusions.

Results. The blood levels found are presented in Table I. In the case of each of the 7 patients with carcinoma of the prostate the mean 17-hydroxycorticosteroid plasma level before stilboestrol and the mean value while taking this drug were calculated. In each of these patients when the mean 17-hydroxycorticosteroid value before stilboestrol is compared with the mean value while taking this drug, a definite increase during the latter period seems obvious. When the means of the means for the group were compared, the 17-hydroxycorticosteroid plasma levels were significantly higher during stilboestrol therapy than before this medication was given $(P = < .01)$. One male with long-standing Addison's disease and maintained on 10 mg oral cortisone acetate twice daily, showed control plasma values of zero when tested on day one and day 3 (the last cortisone was always taken 12 hrs before the blood sample). Stilboestrol 20 mg/day was begun orally on day 3, and 17-hydroxycorticosteroid values of 0.7, 4.5 and 3.8 $\mu g\%$ were found on days 5, 7 and 10 respectively. In the patient with subtotal adrenalectomy, control values were 14.2 and 16.0 $\mu g\%$ while values of 36.9, 35.9, and 40.3 $\mu g\%$ were seen on days 3, 6, and 8 of stilboestrol therapy. The values found on the 2-day ACTH test were 12.4 $\mu g\%$ (before), 18.9 (after) on day one; and 20.2 (before), 21.9 (after) on day 2.

Discussion. The increased levels of plasma 17-hydroxycorticosteroids found in the patients with carcinoma of the prostate while taking stilboestrol might be attributed to (1) interference of the drug with the Porter-Silber color reaction, (2) measurement of a metabolite of stilboestrol, (3) stimulation of the pituitary-adrenal system, (4) interference with normal pathways for removal of adrenal steroids from the blood.

It appears improbable that there was interference with the color reaction since 40 μg of crystalline stilboestrol in chloroform when run through the chromatographic and color developing procedures resulted in a negligible absorption in the range of 410 $m\mu$. Although unlikely because of the benzenoid character of stilboestrol, the possibility exists that some metabolic product of stilboestrol was responsible for the elevated values found. Evidence against this is the small rise produced in the blood levels of the patient with Addison's disease after 7 days on stilboestrol. If a stilboestrol metabolite were produced and measured, a rise similar in magnitude to that seen in the other patients might be expected since all were on the same dose of stilboestrol. Other than male patients with absolute Addison's disease, suitable control patients to test the hypothesis of pituitary-adrenal stimulation would have been males having complete adrenalectomy with no remaining aberrant adrenal tissue, or males with complete anterior pituitary failure (as from hypophysectomy), whose adrenals were not atrophic and would therefore be capable of responding to stimulation. In lieu of such patients, one normally menstruating female (F.P.) who had had subtotal adrenalectomy because of Cushing's syndrome was studied after stilboestrol as well as after ACTH administration. The results were difficult to interpret since they showed normal control levels, poor response to ACTH, and elevated levels after stilboestrol.

The elevated plasma 17-hydroxycorticosteroid values observed following stilboestrol therapy may indeed indicate pituitary-adrenal stimulation. This would be consistent with the evidence in certain animals which indicates pituitary-adrenal activation by estrogens(1,2), but not with the findings of Vogt (3) using hexoestrol in rats. It would support the suggestion of Gemzell(4) that the elevated plasma 17-hydroxycorticosteroid levels found in pregnancy are due to estrogen stimulation of the pituitary-adrenal system. The poor response to ACTH in the patient with subtotal adrenalectomy is not consistent with this theory. The results in this individual suggest that the adrenal remnant was functioning maximally at all times and could hardly be stimulated further by an effect of stilboestrol on the anterior pituitary.

Another hypothesis which would explain the findings in the patients with carcinoma of the prostate is that stilboestrol may interfere with the metabolic pathway normally employed in the removal of 17-hydroxycorticosteroids from the blood, with the resulting accumulation reflected in elevated plasma levels. The data obtained on the patient with subtotal adrenalectomy seem to fit best with this hypothesis; also this hypothesis could account for the results in the patient with Addison's disease. It cannot be concluded from the present data whether stilboestrol stimulated the pituitary-adrenal system in these patients, or whether it blocked a metabolic pathway for disposal of 17-hydroxycorticosteroids.

Summary. Seven patients with carcinoma of the prostate showed a significant rise in plasma 17-hydroxycorticosteroid levels during treatment with stilboestrol as compared with pretreatment control levels. In a male with Addison's disease such a rise was not seen, while in a female with subtotal adrenalectomy a greater rise in these blood levels occurred following stilboestrol than following ACTH administration. Several mechanisms to explain these observations are discussed.

Special thanks are due to Mrs. Angela Cahn and Mrs. Jean Sadler for their technical assistance.

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Increase in Bactericidal Activity as a Result of Injection of Zymosan. (22600)

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In an attempt to isolate one of the components of complement, Pillemer *et al.*(1) separated a protein from normal serum which may have significance in natural immunity. The protein, called properdin, acts only in the presence of complement and Mg^{++} and is capable of destroying bacteria, of neutralizing viruses, and of lysing certain red cells(1,2).

Zymosan,* the insoluble carbohydrate residue from yeast cells, combines with properdin at 17°C in the presence of fresh serum to form a complex which can be removed by centrifugation. Serum deficient in properdin after zymosan treatment resembles normal serum except that the activity against bacteria, viruses, and red cells is greatly diminished or entirely absent(1).

In vivo experiments in mice have demonstrated the ability of zymosan to cause marked changes in susceptibility to experimental *Escherichia coli* infection(2). The ability of zymosan as well as other polysaccharides such as dextran, levan, and glucan to react with properdin(2) suggested the possibility that specific configurations or spatial arrangements are involved and that the injection of one of these polysaccharides into a normal animal would stimulate antibody production to these specific configurations.

Pillemer *et al.*(1) have shown that properdin has a bactericidal action against *E. coli* B. This paper concerns itself with an attempt to increase the cidal activity of rabbit and guinea pig serum for *E. coli* B by means of injections of zymosan incorporated in Freund adjuvants (3).

Methods. Zymosan was suspended in 100 volumes of saline and placed in a boiling water bath for one hour(4). After centrifugation at 3500 rpm for 45 minutes at 0°C, the zymosan was resuspended in a 3.5% solution of fresh egg-white and incubated for 2 hours at 37°C. This mixture was shaken occasionally during incubation and was then taken up in Freund adjuvants(3). As zymosan is a carbohydrate, there is the possibility that it may not be antigenic of itself but may act as a hapten. Therefore, fresh egg-white was added to the zymosan because it has been suggested that antigenic proteins may complex with carbohydrates and impart their antigenic property to the complex(5).

Six guinea pigs and 6 rabbits, all young adult males, were used. Each animal received a subcutaneous injection into the back of the neck, weekly, over a period of 3 weeks. A fourth injection was given 17 days after the third. Each guinea pig received a total of approximately 12 mg of zymosan and each rabbit approximately 24 mg. The animals were bled from the heart before injection and at intervals thereafter. Clotted blood was allowed to remain at room temperature for 2 hours. The serum was then separated in a refrigerated centrifuge and stored in sealed vials in a dry ice chest. The *E. coli* B culture was maintained on nutrient agar slants and transfers made to complete liquid medium(6). After the second serial transfer in this medium, an 18-hour culture was used for inoculum. After 4 or 5 such serial transfers, a new culture was started from the agar slant.

Bactericidal tests were set up in the following manner: Nine ml amounts of complete medium were sterilized in tubes calibrated at 10 ml. Each tube was inoculated with 1 ml containing approximately 1,000 cells of an 18-hour *E. coli* B culture. Sera were added to each tube in the amounts indicated. Mg^{++} was supplied by the addition of $MgCl_2 \cdot 6H_2O$ to the inoculum so as to give a final concentration of 0.07 mg of Mg^{++} ml in the test(7). The volume was made up to the 10 ml mark with distilled water to compensate for loss during sterilization, and after the contents were well mixed, 1 ml amounts were distributed into small sterile tubes. Incubation was at 37°C. At hourly intervals, one tube of each serum concentration was well shaken in a rack to avoid foaming, and pour plates of the entire contents were made in complete agar(6). Plates were incubated for 24 hours and colony counts made.

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* Standard Brands, Inc.

For skin testing, zymosan was prepared in a boiling water bath as previously described, but was centrifuged lightly at 500 rpm for one minute. The very opalescent supernatant was removed and 0.1 ml injected intracutaneously. Boiled as well as formalin treated cells of *E. coli* B were also used in skin tests, 0.1 ml amounts containing 2 x 10^7 and 2 x 10^5 bacteria being injected intracutaneously.

Antigens for agglutination tests were the same boiled and formalinized *E. coli* B suspensions as used for skin tests, but in a concentration of 1 x 10^8 cells/ml. Incubation temperatures of 37°, 46°, and 56°C for 18 hours were all tried.

As not all preparations of zymosan are active, the zymosan used in this experiment was tested for its ability to absorb properdin from normal sera without affecting the hemolytic activity of the serum(4). Rabbit sera were absorbed twice with 5 mg of zymosan/ml at 17°C for one hour each time. The sera were then tested in a standard hemolytic system and found to have retained more than 80% of their original hemolytic activity. Bactericidal activity was found to be greatly diminished, indicating an active zymosan preparation(4).

TABLE I. Bactericidal Activity of Normal and Immunized Rabbit Sera as Determined by Plate Count.

	Viable cells/ml at end of each incubation period					
Hr of incubation	Pool N		Pool G		Pool I	
	Final conc. of serum					
	.75%	1.0%	.75%	1.0%	.75%	1.0%
1	77	79	76	40	5	2
	69	66	63	45	11	3
	92	88	53	46	2	0
	69	63	49	30	6	0
Avg	77	74	60	40	6	1
2	34	43	6	0	0	0
	32	29	12	2	0	0
	58	31	0	6	0	0
	45	33	6	2	0	0
Avg	42	34	6	3	0	0
3	118	65	4	0	0	0
	96	70	20	0	0	0
	260	82	2	4	0	0
	105	46	1	0	0	0
Avg	145	66	7	1	0	0
4	TM*	240	42	4	0	0
		200	94	0	0	0
		250	8	30	0	0
		TM*	20	4	0	0
Avg			41	10	0	0

Pool N: Normal serum pool.
Pool G: Serum pool 7 days after last inj.
Pool I: " " 30 " " " ".

* TM = Too many bacteria to count.

Results. In order to eliminate as far as possible the variables which arise in experiments employing small numbers of bacteria and small amounts of serum, it was found necessary: (a) to report only those experiments in which the inoculum was within the arbitrarily chosen limits of 95 to 105 bacteria/ml; (b) to test in the same experiment, all sera which were to be compared; (c) to repeat the testing of these sera 3 or 4 times at intervals of approximately a week. Table I represents the results of 4 experiments with 0.75% and 1.0% concentrations of normal rabbit serum pool, of rabbit Pool G bled 7 days after the last injection and of Pool I bled 30 days after the last injection. Table II represents 4 experiments with 1.75% and 2.0% concentrations normal guinea pig pool, of guinea pig Pool B bled 11 days after the last injection and of Pool C bled 25 days after the last injection. The hourly average values in Tables I and II clearly indicate a sustained increase in bactericidal activity resulting from the zymosan injections. *Skin test*: Sixteen days after the third injection, the animals were skin tested with the zymosan suspension. There was no reaction at 8 hours, but by 24 hours there was marked reddening. The skin was raised and hard, the central areas were blanched, and later became necrotic. The rabbits showed more severe reactions than did the guinea pigs. Normal animals, skin tested at the same time, showed a slight pink area at 24 hours which had completely faded by 48 hours. Thirty-two days after the last injection (subsequent to the final bleeding), the animals were skin tested with both boiled and

TABLE II. Bactericidal Activity of Normal and Immunized Guinea Pig Sera as Determined by Plate Count.

Hr of incubation	Viable cells/ml at end of each incubation period					
	Pool N		Pool B		Pool C	
	Final conc. of serum					
	1.75%	2.0%	1.75%	2.0%	1.75%	2.0%
1	90	92	84	66	85	84
	95	90	75	94	75	74
	89	85	83	82	65	57
	79	67	78	73	78	68
Avg	88	84	80	79	76	71
2	104	124	15	2	4	0
	130	90	12	11	3	0
	139	75	4	5	1	0
	140	85	10	0	4	2
Avg	128	94	10	5	3	1
3	280	360	5	1	0	0
	410	210	4	6	0	0
	360	140	0	4	0	0
	440	170	2	0	0	0
Avg	376	220	4	3	0	0
4	TM*	TM*	14	0	0	0
			0	2	0	0
			0	4	0	0
			5	0	0	0
Avg			5	2	0	0

Pool N: Normal serum pool.
Pool B: Serum pool 11 days after last inj.
Pool C: " " 25 " " " ".

* TM = Too many bacteria to count.

formalinized suspensions of *E. coli* B cells. The experimental animals failed to show appreciable differences from the normals.

Agglutination tests with *coli* antigens and rabbit and guinea pig sera failed to show consistent differences between normal and injected animals.

Rabbit Pool I, bled 30 days after the last injection, was absorbed with 5 mg of zymosan/ml of serum at 17°C for one hour. After centrifugation at 3500 rpm for 45 minutes at 0°C, this serum lost none of its hemolytic activity but showed a marked decrease in bactericidal potency. A second absorption of the same serum resulted in some 25% loss of hemolytic activity and a further loss in cidal activity (Table III).

Discussion. The increase in bactericidal titer, the positive Arthus phenomenon, and the ability to absorb bactericidal activity with zymosan, all suggest that antibodies were produced as a result of immunization with zymosan. Thus the possibility exists that antibody against zymosan possesses receptor sites capable of reacting with a specific polysaccharide configuration; a configuration which may be present in *E. coli* B, viruses, and red cells. The concept of the antigenicity of zymosan may be carried a step further to help explain a number of experiments(8-12) which have recently appeared in the literature. Workers have injected substances such as levan(8), lipopolysaccharides(9), typhoid vaccine(10), and zymosan(10-12) into mice and found a rapid fall in properdin level followed in 3 to 6 days by a rise to a level greater than normal. After about 10 to 14 days, the properdin level returned to normal. This fall and rise in properdin was paralleled by the degree of susceptibility of the mice to infection(8-12). Thus it would seem that there may be groupings on levan, lipopolysaccharides, typhoid vaccine, zymosan, *E. coli* B, viruses and perhaps other substances which can combine with receptor sites on properdin. The fact that the properdin level falls rapidly on injection of the above substances coincides with what would be expected if one injected antigen into an immune animal; namely, a rapid fall in antibody followed by a subsequent rise and then a downward levelling off. The response of properdin in the serum of a normal animal to injections of levan, zymosan, etc., is very much like the antibody response of the immune animal to specific antigen.

Landsteiner(5) in his discussion of natural antibodies concluded that normal serum contains substances which are formed indepen-

TABLE III. Bactericidal Activity* of Pool I Rabbit Serum after Absorption with Zymosan as Determined by Plate Count.

Hr of incubation	Absorbed once		Absorbed twice	
	.75%	1.0%	.75%	1.0%
1	91	85	88	87
2	97	40	172	183
3	300	68	TM†	TM†
4	TM†	TM†	TM†	TM†

After first absorption serum lost no hemolytic activity.

After second absorption serum lost 25% of hemolytic activity.

* Due to lack of serum, figures represent avg of only 2 duplicate experiments.

† TM = Too many bacteria to count.

dently of external antigenic stimuli, have a low order of specificity and resemble the antibodies produced by immunization. It is proposed that the properdin fraction of serum contains these normal substances which destroy bacteria, neutralize viruses, and destroy red cells, and that the injection of zymosan stimulates the production of antibodies with receptor configurations similar to those found in the properdin of normal serum. Such a proposal would readily explain the sustained increase in bactericidal activity of the serum due to the presence of small amounts of zymosan, also the ability of zymosan to absorb bactericidal activity due to immunization as well as to properdin.

Kabat and Berg(13) in demonstrating the antigenicity of dextran in man, suggested that "In all probability, other polysaccharides composed of only a single sugar, levans, mannans, etc., will also prove to be antigenic in man —". Zymosan belongs to this group of polysaccharides and it may be possible, by injecting zymosan, to increase the "natural antibodies" in the human species.

Summary. 1. Bactericidal activity against *Escherichia coli* B was increased in rabbits and guinea pigs as a result of injections of zymosan mixed with egg-white and incorporated in Freund adjuvants. 2. These rabbits and guinea pigs responded to intracutaneous injection of zymosan with the Arthus phenomenon. 3. Zymosan absorbed the bactericidal activity without affecting the hemolytic activity of the sera. 4. The possible antigenicity of zymosan is discussed.

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Turbidimetric Determination of Proteins with Sulfosalicylic and Trichloracetic Acids. (22601)

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Folin and Denis(1), using a method proposed earlier for the nephelometric determination of protein in milk and digestion mixtures, introduced the turbidimetric determination of protein in urine with sulfosalicylic acid (SA). Subsequently, this technic was extended to protein determination in spinal fluid by Denis and Ayer(2) and in serum or plasma by Looney and Walsh(3). Mestrazat(4) used trichloracetic acid (TCA) for the turbidimetric determination of protein in spinal fluid.

The biuret reaction has almost completely supplanted turbidimetry for the routine determination of protein in serum in the clinical laboratory, but because of its greater sensitivity the turbidimetric method is still widely used for urine and spinal fluid and most textbooks advise the use of SA.

Although some workers(3,5) have reported observing no difference in the turbidity produced with albumin or globulins by SA, Plötner(6) claimed that by nephelometry the turbidity produced by SA with albumin was about 4 times that with globulin. Bossak and coworkers(7) found that, by turbidimetry, albumin gives about twice the turbidity given by γ-globulin, whereas much better agreement was found using TCA. They, therefore, recommended the use of TCA for the turbidimetric determination of protein in spinal fluid. The difference in turbidity produced by SA with albumin and globulin was again confirmed nephelometrically by Heepe and coworkers(8). Their results, however, showed a parallel difference with TCA.

Obviously, if the degree of turbidity produced is dependent on the relative concentration of albumin and the various globulins, quantitative results are subject to an uncontrollable, and in most instances unknown, variable. This study compares results obtained using SA, TCA, and a mixture of the 2 acids (SA and TCA) giving identical turbidities with albumin and γ-globulin, on a series of sera with a wide range of A/G ratios.

Materials and methods. Human albumin (Lot No. 940 C2B, Hyland Laboratories, Los Angeles, Calif.) and human γ-globulin (Lot No. 2175-249-B, Poliomyelitis Immune Globulin, Lederle Lab. Division, American Cyanamid Co., New York City) were standardized by Kjeldahl N and in turn were employed for standardizing the biuret test and determining relative turbidities produced by SA, TCA and the SA-TCA mixture. The biuret method was employed as the reference method for total protein since biuret equivalents of the various protein components of serum are identical or very nearly so, thus accounting for the close agreement with results obtained by Kjeldahl N determination(9). Precipitation of globulins for determination of the A/G ratio was carried out by the method of Wolfson and coworkers(10). Sera were diluted 1:600, 1:400 and 1:600 with 0.85% NaCl for use with SA, TCA and SA-TCA, respectively. To 4.0 ml of these dilutions was added 1.0 ml of the acid reagent followed by immediate mixing. The SA and TCA reagents were 12.5% and the SA-TCA reagent was a mixture of 3 volumes of the SA reagent and 4 volumes of the TCA reagent. The turbidities were read between 5 and 10 minutes after addition of reagents and after remixing and allowing any air bubbles thereby produced to rise to the surface, in a Bausch and Lomb Spectronic 20 at 420 mμ, using the square cuvettes (light path of 11.5 mm) and water as a blank. The experiments were carried out at room temperature which was 24 ± 2°C.

Results. Acid Concentration. Final concentrations of 2.5 and 5.0% of TCA, SA and the SA-TCA mixture were compared. Because of the greater tendency towards flocculation of the protein precipitate with the higher acid concentration, lower absorbence (A) readings were obtained in general. It was concluded that the finer suspension produced by the lower acid concentration was preferable. *Time of reading.* Albumin consistently flocculated more quickly than γ-globulin. Measurements made on albumin-γ-globulin mixtures and several sera at 1 minute intervals up to 20 minutes showed fairly consistent readings between 5 and 10 minutes after the addition of the acid. If readings are delayed until gross flocculation occurs it is difficult to obtain proper dispersion, a prerequisite to a correct reading. *Gum ghatti.* Use of gum ghatti as a protective colloid(3) was found to delay but not prevent flocculation. Occasionally, its presence appeared to inhibit the development of turbidity. Since no particular advantage was apparent from its use, and perhaps a disadvantage, it was discarded. *Reproducibility.* The 95% limits at a mean absorbence of 0.370 were found to be ± 5% from the mean. *Relative turbidities of human albumin and γ-globulin.* Standardization curves with solutions of these 2 proteins in 0.85% NaCl, using SA, TCA and the SA-TCA mixture, are shown in Fig. 1. One ml of the acids was added to 4 ml of the protein concentrations indicated. With SA, the turbidity given by albumin was about 2.4 times that given by γ-globulin. With TCA, the globulin gave about 1.2 times the turbidity given by albumin. Since the differences produced by these two acids were in opposite directions, a successful search was made by trial and error for a combination of the acids which gave the same turbidities with both

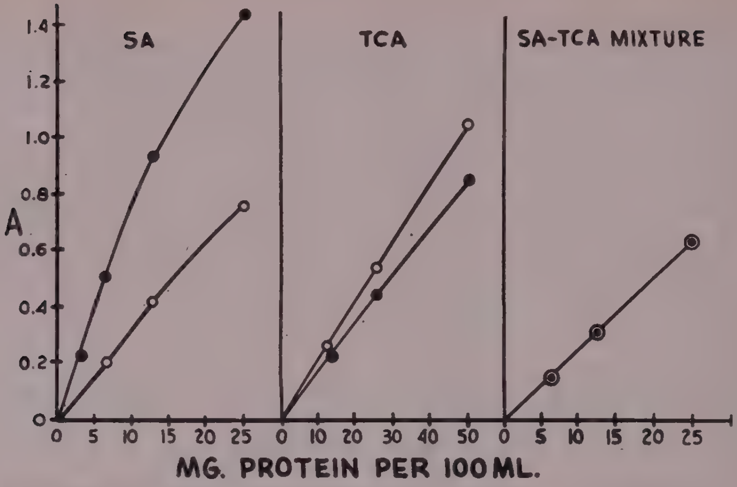

FIG. 1. Standardization curves of human albumin and γ-globulin. ● = albumin; ○ = γ-globulin.

proteins. *Results with sera of varying A/G ratios.* Results obtained with 67 sera are shown in Fig. 2 in which the absorbence values obtained are plotted *vs.* the serum protein concentration. The regression lines shown were calculated as y on x. The correlation coefficients, r, are indicated on the respective graphs. The r values for TCA and the SA-TCA mixture are both significantly greater than that for SA (t test, $P<0.02$). The r value for the SA-TCA mixture, however, is not significantly greater than that of TCA.

To detect the possible dependence of the degree of turbidity produced on the A/G ratio, 100 times absorbence/total protein is plotted for the same series of sera *vs.* the A/G ratio in Fig. 3. The regression coefficients, b for y on x, are shown on the respective graphs. The b value for SA is significantly greater than O (F test after analysis of variance of scatter from regression line, $P<0.001$), indicating a dependence of results on the A/G ratio. As predicted from the results with albumin and γ-globulin, the slope is positive. The b values for TCA and the SA-TCA mixture are not significantly different from O, giving no indication of a dependence with these acids on the A/G ratio.

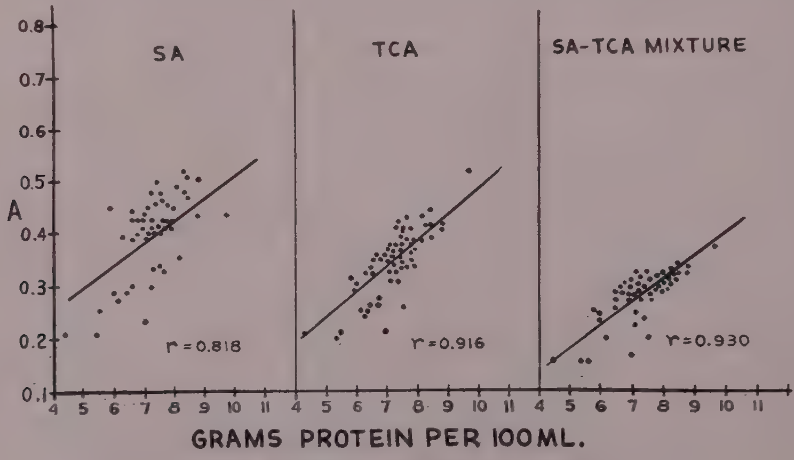

FIG. 2. Scatter diagrams of turbidities produced by 67 sera.

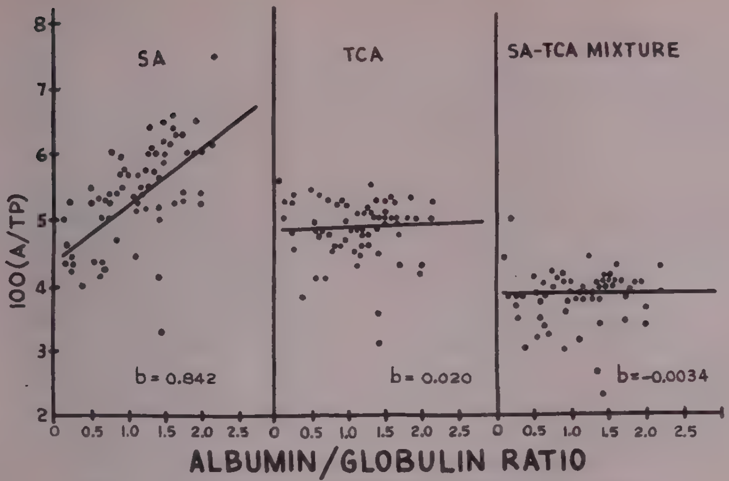

FIG. 3. Dependence of turbidities produced on A/G ratio.

Deviation from the average value of 100 times absorbence/total protein constitutes an estimate of the accuracy of the turbidimetric method. The 95% limits (2 coefficients of variation) for SA, TCA and the SA-TCA mixture are ± 27.2, ± 20.4, and ± 22%, respectively.

Discussion. The estimated accuracy of the turbidimetric method is not very high, being at best about ± 20%. Whether or not this is sufficient for the clinician is beyond the scope of this discussion. The TCA and SA-TCA reagents gave results of significantly greater accuracy than the SA reagent, but improvement was not as great as was expected. There is little doubt that the SA results are correlated with the A/G ratio and thus are subject to an uncontrolled variable. It is quite possible that the turbidities produced by the various proteins other than albumin and γ-globulin present in body fluids are not equal using TCA or the SA-TCA mixture, thus accounting at least partly for the relative inaccuracy observed. Reports by some workers(3,11) of close agreements between results obtained by SA and Kjeldahl N are difficult to understand. Inasmuch as no disadvantage is apparent, but rather a significant advantage, it is believed advisable to supplant SA with TCA for routine turbidimetric determinations. Although a SA-TCA mixture was found which gave better results with albumin and γ-globulin than TCA, results with sera gave no evidence of superiority.

Summary. The turbidity produced by addition of sulfosalicylic acid to a solution of a mixture of proteins is correlated with the A/G ratio. No correlation is apparent when trichloracetic acid is employed. It is suggested, therefore, that trichloracetic acid should supplant sulfosalicylic acid in turbidimetric methods for the determination of protein.

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Antihypertensive Action of Neotetrazolium. (22602)

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Tetrazolium salts have been used to measure various aspects of the dehydrogenase activity in living tissue systems. Most experiments have dealt with excised structures(1,2) or with tissue homogenates(3) incubated *in vitro*. The reaction between the tetrazoles and living enzyme systems involves reduction of the colorless salt to an insoluble purple to black formazan, with the greatest accumulation in areas of growth, multiplication and high metabolic activity(1). It has also been shown that pp′-diphenyl bis 2-(3,5 diphenyl) tetrazolium chloride (Neotetrazolium), when injected intraperitoneally or subcutaneously in the monkey, rat or rabbit, lodges in considerable amount in the cerebrospinal and sympathetic ganglia of the nervous system where it is reduced to the insoluble formazan particularly in the satellite cells, but also in the nerve cells. The darkly colored ganglia stand out prominently in contrast to the remainder of the nervous system which remains uncolored(2,4).

Since it has been shown that blood pressure can be reduced in hypertensive conditions by interfering either by surgical or pharmacological means with nerve transmission through the sympathetic ganglia, the possibility was entertained that the intracellular deposition of tetrazolium salts might produce ganglionic blockade. Accordingly, the effect of these salts on the blood pressure of hypertensive rats was investigated.

Methods. Neotetrazolium (NT) was the agent used, hypertensive rats receiving daily subcutaneous injections of 0.5 ml or 0.25 ml of a 0.5% solution in saline. The studies were carried out on a series of 12 rats made hypertensive either by a figure-of-eight loop about both kidneys, or unilateral nephrectomy and constriction of the remaining renal artery. The hypertension was allowed to develop for periods of from 2-6 months, before placing the rat on the tetrazolium regime. Blood pressures were recorded by the Friedman microphonic method(5) on the tail of lightly etherized rats.

Experimental. In most instances the NT was administered for test periods of from 5-10 days. After 3 days of treatment, the blood pressure began to fall and remained at hypotensive levels as long as the NT was administered. On the average, the blood pressure was reduced by 90-100 mm Hg, reaching levels as low as 60-70 mm Hg. The lowest level was reached within 3-5 days after the initial NT injections and the pressure remained in this range for an additional 2-3 days after terminating treatment. Thereafter, the blood pressure rose and within a week had returned to its previous high level. In 2 of the rats, the blood pressure did not return to its original hypertensive levels for at least a month.

Fig. 1 is a representative protocol which shows the rapid decline in mean blood pressure after daily administration of 0.5 ml of the NT solution to a 4-month hypertensive rat. As indicated, a second series of 4 injections with 0.25 ml of the NT solution likewise had a dramatic antihypertensive action.

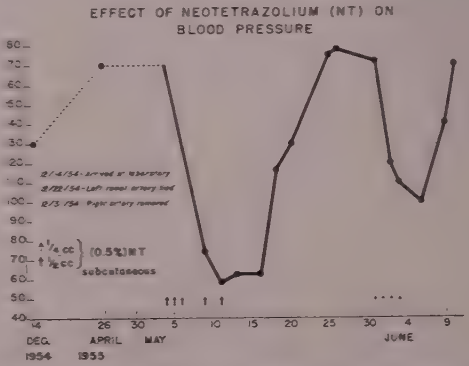

FIG. 1. Representative protocol of blood pressure measurements (indirect) of renal hypertensive rat (partial ligation of renal artery). Each point on B.P. curve represents avg of 4-5 readings at indicated interval. (C. F. Wistar strain, female, wt at initial operation 140 g, at final reading 210 g.)

Although injection of NT, in the amounts used, resulted in a moderate loss of weight (20-30 g), the animals appeared in good health. In addition to the formazan present in the ganglia, various other tissues showed evidence in coloration. The adrenals were lightly colored, with the formazan chiefly in the innermost reticularis. The kidney and liver had a faint purplish hue, which on histological examination showed formazan deposits in the parenchymal cells. In the brain proper, no reduced NT was evident in section including the hypothalamus and vasomotor centers.

A small series of normotensive controls (4 rats) were placed on a comparable NT regime and blood pressure measurements taken. The larger dose, 0.5 ml of a 0.5% NT solution, dropped the pressure from control levels of 110-120 mm Hg to 60-65 mm Hg. The smaller dose 0.25 ml of 0.5% NT produced only a transient and fleeting drop of 20-30 mm in blood pressure for 2-3 days when administered for 6 consecutive days.

Discussion. The mechanism whereby NT reduces the blood pressure of hypertensive rats is not clear. The fact that NT is irreversibly reduced to formazan in the nervous system ganglia suggests a chemical blocking action equivalent to temporary ganglionectomy. It is unlikely that the physical presence of formazan *per se* is responsible for the fall in blood pressure since the deposits remain in the ganglia almost indefinitely, while the blood pressure returns to hypertensive levels in about 5 days. The possibility therefore remains that with the irreversible reduction of NT, enzyme activity is impaired(3,6). Black, Zweifach and Speer(7) have shown that sodium azide, a metabolic inhibitor, produces a lowering of blood pressure in rat and man with hypertension.

Inasmuch as the tetrazolium salts are also deposited in the adrenal cortex, particularly in the reticularis, following subcutaneous injection(1,2), the role of an adrenal pathway is likewise to be considered. In view of the minor *in vivo* deposition of NT in the kidneys, the possible interference with the elaboration of a renal factor appears unlikely, although not ruled out.

Summary. When the tetrazolium salt pp'-diphenyl bis 2-3 (3, 5 diphenyl) tetrazolium chloride (Neotetrazolium) is injected subcutaneously, it lodges in considerable amount in the sympathetic ganglia of the nervous system where it is reduced to the insoluble formazan. The satellite cells are heavily colored in contrast to the nerve cells. A series of renal hypertensive rats were given daily subcutaneous injections of from 0.25-0.5 ml of a 0.5% solution of neotetrazolium (NT) for periods of from 5-10 days and the blood pressure recorded by an indirect microphonic method on the tail. The NT lowered the blood pressure within 2 days after the treatment was initiated to levels as low as 60-70 mm Hg. Within 1 week after treatment, the pressure had returned to control levels. Subsequent injections had a comparable hypotensive action. It is suggested that NT may reduce the blood pressure by impairing the metabolic activity of the ganglionic cells, although an indirect effect on other organs such as the adrenal has not been ruled out.

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Lack of Effect of Dietary Choline on Cholesterol Absorption in Rat.[*] (22603)

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Two recent reports indicate that dietary choline influences plasma cholesterol levels in the rat. Weiss *et al.* found that rats fed a diet containing cholesterol and low in lipotropic factors exhibited plasma cholesterol levels markedly lower than animals fed the same diet supplemented with choline and inositol(1). Ridout *et al.* reported that dietary choline further accentuated the rise in plasma cholesterol occurring 2 to 4 hours after cholesterol feeding(2). These authors suggested that this effect of choline may be due to some action on cholesterol absorption or utilization.

Consideration of these findings has led to the working hypothesis that choline may stimulate cholesterol absorption, and the experiments here described were designed to test this hypothesis. The results indicate that under the conditions reported, choline had no significant effect along these lines.

TABLE I. Composition of Diets.[*]

Ingredients	Diet A (%)	Diet B (%)
Casein	10	15
Sucrose	72	56
Cottonseed oil	9.5	20
Celluflour	2	4
Salt mixture[†]	5	4.5
L-Cystine	0.5	0.5
Inositol	1.0	0.1
Vitamins	[‡]	[‡]

[*] The supplemented diets contained the following amounts of choline chloride: Diet A, 1%; Diet B, 0.5%.

[†] Wesson modification of Osborne-Mendel salt mixture.

[‡] Each kilogram of diet contained following vitamins: 10 mg each of thiamin hydrochloride, riboflavin, and pyridoxine hydrochloride; 60 mg calcium pantothenate, 60 mg niacin, 0.1 mg biotin, 400 mg para-aminobenzoic acid, 10 mg folic acid, 100 mg ascorbic acid, 5 mg menadione, 4000 USP units vit. A, 800 USP units vit. D, and 0.5 g mixed tocopherols. Water-soluble vitamins were mixed with sucrose, fat-soluble ones with cottonseed oil, before being added to the diet.

Methods. A. *Direct determination of cholesterol absorption by thoracic lymph collection.* Male Long-Evans rats, weighing from 90 to 120 g, were fed synthetic diets deficient in, and supplemented with, choline (Diet A, Table I). After a minimum feeding period of 3 weeks, thoracic lymph ducts were cannulated by a modification of the technic of Bollman *et al.*(3). The rats were given 0.85% saline to drink in order to encourage lymph flow. After 24 hours, from 3 to 5 mg of cholesterol-4-C^{14}[‡] dissolved in 0.5 to 0.7 ml of Wesson oil were administered by stomach tube to animals exhibiting satisfactory lymph flow, and lymph was then collected in graduated centrifuge tubes for one or 2 hour intervals over a period of 12 to 24 hours. Aliquots of the lymph were placed directly on tared aluminum counting dishes, dried under a heat lamp, and the radioactivity measured in a gas flow counter. The results were corrected for self-absorption. The cholesterol absorption was computed from the radioactivity recovered in the lymph at the end of twelve hours and expressed as percent of the radioactivity administered.

B. *Indirect determination of cholesterol absorption by analysis of gastro-intestinal contents.* Male weanling rats of the University of Southern California strain were fed diets deficient in, and supplemented with, choline (Diet B, Table I) for a period of 6 weeks. Following a 24-hour fast, 1.6 mg of cholesterol-4-C^{14} dissolved in 1 ml of Wesson oil were administered by stomach tube to each rat under light ether anesthesia. At the same time, the supplemented rats received, by the same route, 25 mg of choline chloride dissolved in 0.25 ml of water. After a 6 hour absorption period, the animals were sacrificed

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[*] Supported in part by research grant H-1162 from the National Heart Institute, National Institutes of Health, Public Health Service.

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[‡] The cholesterol-4-C^{14} was prepared and purified as reported(4); it was homogeneous when analyzed by paper chromatography(5) and had a specific activity of 1.3×10^5 cpm/mg of C (gas flow counter).

TABLE II. Influence of Dietary Choline on Cholesterol Absorption as Determined Using Rats with Lymph Fistulae.

Choline supplementation	No. of rats	Radioactivity in lymph at 12 hr as % of radioactivity administered	
		Mean	Range
1% of diet	6	18.4	11.8–25.2
None	6	26.7	18.7–43.3

with ether; the livers were removed immediately and weighed.§ The gastrointestinal tract was excised and divided into stomach, upper intestine and lower intestine. The contents of each of these sections were collected separately by rinsing three times with 20 ml portions of hot saline. The washed walls of the intestine, the liver, and in some cases the carcass were also analyzed. Ethanol and potassium hydroxide were added to each sample to a final concentration of 50% (v/v) and 30% (w/v), respectively. The mixtures were heated for 2 hours to complete hydrolysis and then extracted 5 times with petroleum ether (Skelly B). The combined extracts of each sample were evaporated to dryness. The residues were dissolved in chloroform containing 75 mg of Wesson oil per ml of solvent. Duplicate aliquots were placed on tared counting dishes provided with lens paper to prevent creeping. After evaporation of the solvent, radioactivity was determined as above. Cholesterol absorption was computed by subtracting the radioactivity of the intestinal contents from the administered radioactivity and expressed as percent of the administered dose. In general, this difference agreed well with the sum of the radioactivities recovered from the liver, washed walls of the gut, and the carcass.

Results. The results of the first experiment (lymph collection) are presented in Table II. It can be seen that variation among individual animals was considerable. The values for the 2 groups overlap, and statistical analysis fails to show a significant effect of dietary choline. (P for difference of means: between 0.5 and 0.1). It is possible that these variations were in part a consequence of the use of surgery and restraining cages, both of which might affect absorption by way of the nervous and circulatory systems(6). The second experiment was undertaken in an attempt to eliminate these two possible sources of variability. No surgery was performed, and except for the slight trauma of intubating the radioactive cholesterol, the rats were not disturbed. In addition, weanling rats were used, and changes were made in the basic diet in order to produce a more severe choline deficiency, and, at the same time, promote better health and growth (Table I). The amount of choline in the supplemented diet was reduced, since the higher level fed in the first experiment appeared to cause anorexia. The results of the second experiment are given in Table III. The rats fed the deficient diet exhibited markedly enlarged, fatty livers (mean weight, as % of body weight: 6.68; control group: 3.27). One animal which died on the eighth day of the preliminary feeding period showed hemorrhagic kidneys. Both of these symptoms are typical of severe choline deficiency. As in the first experiment, cholesterol absorption was highly variable, and a statistical analysis of the values obtained indicates that

TABLE III. Influence of Dietary Choline on Cholesterol Absorption as Determined from Radioactivity of Gastrointestinal Contents of Rats Fed Cholesterol-4-C^{14}.

Choline supplementation	No. of rats	Mean radioactivity recovered at 6 hr as % of radioactivity administered*			
		GI contents	Washed intestinal wall	Liver	Carcass
.5% of diet	9	45.4 (29.2-62.4)	45.9 (36.1-53.4)	5.6 (.2-10.4)	.2† (.0-.4)
None	9	51.0 (32.2-79.8)	39.6 (21.4-57.4)	4.9 (1.4- 9.5)	.0† (.0-.0)

* Ranges are given in parentheses. † Two animals only.

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§ Since, in the first experiment, most of the absorbed cholesterol did not reach the blood stream, and as, in the second experiment, the quantities of sterol administered and ingested with the diet were too low to significantly influence plasma cholesterol levels, the latter values were not determined.

there is no significant difference between the 2 groups of animals. (P for difference of means: between 0.4 and 0.5). It was concluded that, under the conditions of these experiments, choline had no significant effect on the intestinal absorption of cholesterol. These results appear to parallel findings of Tasker(7) and of Shoshkes *et al.*(8), who observed that choline did not influence the absorption of olive oil, and corn oil respectively, using technics similar to those reported in this paper.

Summary. The effect of dietary choline on the intestinal absorption of cholesterol was studied by 2 different technics. The results indicate that under the conditions of these experiments, choline did not influence cholesterol absorption.

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Effect of Exposure to a Cold Environment on Labeling of Phospholipide in Rat Liver Slices.* (22604)

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If rat liver slices are allowed to respire in a suitably buffered medium containing acetate-1-C^{14}, the labeled carbon atom of the acetate is incorporated into the acetone-insoluble lipide, chiefly phospholipide(1,2). Recently Kline, DeLuca and Rossiter(3) reported that the labeling of the phospholipide of rat liver slices was decreased if the animal was exposed to a cold environment for either short (3 hr. at —5°C) or longer (12 days at 4-5°C) periods of time prior to killing.

In the present paper the effect of cold exposure on the subsequent labeling of the acetone-insoluble lipide of rat liver slices is described for a number of radioactive precursors. Inorganic P^{32}, acetate-1-C^{14}, glycerol-1-C^{14} and glycine-2-C^{14} were chosen for comparison, for it has been shown that each of these precursors labels a different portion of the phosphoglyceride molecule(4).

Methods. Male rats of the Sprague-Dawley strain (150-200 g) were maintained in an air-conditioned room at 22°C. In one series of experiments, groups of rats were transferred to a cold room maintained at 4-5°C for the 12 days preceding the experiment. In other experiments, groups of rats were transferred to a cold room maintained at —5°C for the 3 hours immediately prior to killing. In each experiment, groups of control animals maintained at 22°C were studied at the same time. All animals were fed *ad libitum* Master Fox Cubes (Toronto Elevators, Ltd.), containing approximately 53% carbohydrate, 3.5% fat and 20% protein.

Each animal was killed by decapitation. The liver was perfused rapidly *in situ* with cold (2-5°C) isotonic saline. Slices were prepared free-hand and approximately 500 mg sliced tissue was incubated for 4 hours at 37.5°C in a Warburg vessel containing Krebs-Ringer bicarbonate buffer. For the C^{14} ex-

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TABLE I. Effect of Exposure to a Cold Environment (12 Days at 4-5°C) on Subsequent Labeling of Acetone-Insoluble Lipides (Phospholipide) of Rat Liver Slices.

		Specific activity (c.p.m./mg lipide)		Decrease	
Exp. No.	Precursor	Control (22°C)	Cold (4-5°C)	(%)	P
1.	Acetate-1-C^{14}	5190 ± 560* (4)†	910 ± 120 (6)	82	$<.001$
2.	Inorganic P^{32}	150‡ ± 6 (7)	140‡ ± 9 (6)	7	$>.5$

* Mean ± stand. error of mean. † No. of animals. ‡ Counts/min./μg P.

periments 3 μmoles labeled substrate containing 1.3 μc radioactivity was added to the medium. For the P^{32} experiments 24 μc carrier-free inorganic P^{32} was added. At the end of the incubation period the tissue was homogenized and the lipides extracted. The phospholipides were precipitated and the radioactivity determined as described by Kline and DeLuca(*2*) for the C^{14} experiments and as described by Strickland(*5*) for the P^{32} experiments. Details of the preparation of the tissue, incubation, extraction and purification of the phospholipide, and counting procedures have been published(*2*).

Results. Table I shows that exposing rats to an environmental temperature of 4-5°C for 12 days caused a great reduction in the labeling of the acetone-insoluble lipide when acetate-1-C^{14} was the precursor, thus confirming previous findings(*3*). Table I also shows that a similar cold exposure had no significant effect on the labeling of phospholipide from inorganic P^{32}. When the rats were exposed to —5°C for the 3 hours immediately preceding the experiment, there was a decrease in the labeling of the acetone-insoluble lipide from acetate-1-C^{14}, again confirming previous findings (Table II)(*3*). However, when the radioactivity was derived from glycerol-1-C^{14}, glycine-2-C^{14}, (Exp. 3) or inorganic P^{32} (Exp. 4), there was no such significant decrease.

Discussion. The results indicate that previous exposure of rats to a cold environment causes a decrease in the subsequent labeling of the acetone-insoluble lipide of liver slices when acetate-1-C^{14} is the source of the radioactivity, but no significant change when the radioactivity is derived from glycerol-1-C^{14}, glycine-2-C^{14} or inorganic P^{32}.

Pihl and Bloch(*1*) showed that the fatty acid portion of the acetone-insoluble lipide was labeled when acetate-1-C^{14} was the precursor. Hydrolysis of the phospholipide of rat liver slices has confirmed this finding and has also shown that, with this precursor, the glycerol and the nitrogen-containing moieties of the phosphoglyceride molecule are not labeled to any significant extent(*4*). On the other hand, hydrolysis experiments, using the chromatographic procedures of Dawson(*6*), have shown that glycerol-1-C^{14} labels the

TABLE II. Effect of Exposure to a Cold Environment (3 Hr at –5°C) on Subsequent Labeling of Acetone-Insoluble Lipides (Phospholipides) of Rat Liver Slices.

		Specific activity (c.p.m./mg lipide)		Decrease	
Exp. No.	Precursor	Control (22°C)	Cold (–5°C)	(%)	P
3.	Acetate-1-C^{14}	3130 ± 380* (6)†	1610 ± 350 (6)	49	$<.02$
	Glycerol-1-C^{14}	1310 ± 130 (6)	1240 ± 160 (6)	5	$>.7$
	Glycine-2-C^{14}	1910 ± 120 (6)	1830 ± 60 (6)	4	$>.5$
4.	Inorganic P^{32}	140‡ ± 9 (6)	130‡ ± 4 (6)	7	$>.05$

* Mean ± stand. error of mean. † No. of animals. ‡ Counts/min./μg P.

glycerol portion of the phosphoglyceride molecule, but neither the base nor the fatty acid(4). Moreover, glycine-2-C^{14} labels the base (particularly the serine of phosphatidyl serine and the ethanolamine of phosphatidyl ethanolamine), but does not label to any significant degree the glycerol or the fatty acid portion of the molecule(4). It would thus appear that previous exposures of rats to a cold environment causes a decrease in the subsequent turnover of the fatty acid portion of the phospholipide in respiring liver slices, with no change in the turnover of other portions of the molecule. The finding that, with acetate-1-C^{14} as precursor, cold exposure causes a decrease in the labeling of the fatty acid portion of the phospholipides of rat liver slices, taken in conjunction with the report of Masoro, Cohen and Panagos(7) that under similar conditions there is a decrease in the labeling of the total fatty acid, might suggest that after cold exposure there is some decrease in the ability of the liver slice to metabolize acetate. However, the observations that cold exposure causes no significant decrease in the incorporation of labeled acetate into cholesterol(3,7), or in the oxidation of labeled acetate to CO_2(7) indicates that any change in acetate metabolism must be subsequent to its activation to form acetyl-CoA.

Summary. In rat liver slices respiring in a buffered medium containing suitable radioactive precursors, the acetone-insoluble lipide (phospholipide) is labeled metabolically. Exposure of the animals to an environmental temperature of 4-5°C for the 12 days prior to the experiment caused a decrease in the subsequent labeling of the phospholipide from acetate-1-C^{14}, but no significant change when inorganic P^{32} was the source of the radioactivity. Exposure of the rats to —5°C for the 3 hours immediately prior to the experiment caused a decrease in the labeling of the phospholipide when the radioactivity was derived from acetate-1-C^{14}, but no change when it was derived from glycerol-1-C^{14}, glycine-2-C^{14} or inorganic P^{32}.

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Effects of 6-Mercaptopurine on Respiration, Anaerobic Glycolysis and Succino-Dehydrogenase Activity in Normal and Tumor Tissues.[*]

(22605)

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It has been reported that 6-mercaptopurine (6-MP) produces a characteristically prolonged inhibition of the Crocker mouse Sarcoma 180 (S-180). Immediately after treatment with effective doses the majority of tumors fail to "take" when reinoculated into normal mice. Moreover, the survival of treated mice is extended and in a significant number tumors disappear completely. In the latter animals the loss of tumors begins as a resorptive process one to 3 weeks after the end of therapy and is completed within another one to 2 weeks(1). Characteristic cytological changes are also seen in treated S-180. There is a predominance of giant, multinuclear cells with both nuclear and cytoplasmic vacuolization. Some of the aberrant cells show irregular, stellate cytoplasmic processes and contain peripherally located, basophilic nuclei. These cytological alterations appear to persist until resorption is complete or growth is resumed.

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In spite of the cellular lesion produced by 6-MP, treated S-180 is free of necrosis and is viable by all accepted pathologic criteria. The evidence suggests that the lesion is associated with a persistent metabolic alteration which does not immediately lead to cell-death. To examine this possibility more critically we undertook a study of the respiration, anaerobic glycolysis, and succino-dehydrogenase activity of S-180 and of certain normal tissues of treated mice.

Methods. Mice with S-180 were treated with growth inhibitory doses of 6-MP as previously described(1). Tumors, livers and kidneys were studied 12 hours after the end of treatment. The animals were sacrificed by cervical dislocation and the tissues were placed in iced, buffered Krebs-Ringer solution(2). Slices were prepared free-hand by razor and were introduced into Warburg flasks; they were thin enough so that respiration was not limited by oxygen diffusion. The dry weight of tissue in each flask was about 16 mg in the respiration experiments and about 8 mg in the anaerobic glycolysis experiments. The fluid volume was 3.0 ml in the respiration experiments: 0.2 ml KOH 20% in the central well and 2.8 ml Ringer-phosphate buffer, pH 7.0, in the main well. In the anaerobic glycolysis experiments 2.0 ml of Ringer-bicarbonate buffer with 0.2% glucose (pH 7.1) were used in the main well. In a few experiments neutralized horse serum was used which was prepared as follows: 20 ml of serum were added to 3 ml of HCl, 0.109 N. Evacuation of CO_2 from the acidified serum resulted in a pH of 7.4. Thereafter 2 ml of M/15 phosphate buffer, pH 7.4, were added. The gases used, O_2 and N_2-CO_2 mixture, were flushed through flasks for 10 minutes. The N_2-CO_2 mixture was passed through glowing copper wool before introducing into flasks. Equilibration time was 20 minutes, and readings were made every 10 minutes for 90 minutes following the end of equilibration. Since the values obtained in each reading were uniform throughout the experimental period, $Q\,O_2$ and $Q\,{}^{N}_{CO_2}$ were calculated on the basis of the total gas displacement. Measurements of succino-dehydrogenase activity were performed on tissue homogenates according to the Schneider-Potter technic(3).

Results. The effects of 6-MP on respiration of S-180 and of liver and kidney were first studied in mice treated intraperitoneally with 50 mg/kg/day for 7 days beginning one day after tumor transplantation. In a few instances the respiration of liver and kidney was also measured in normal animals receiving similar doses of the agent. The results presented in Table I show that treatment decreased the QO_2 of tumor tissue to about 45% of control values though it did not significantly alter QO_2 values of liver and kidney. An analysis of variance revealed that the difference in respiration of S-180 due to treatment was significant beyond the 1% level of probability ($F = 39.11$; p. $0.01 = 11.26$). Although control values varied on different days for unexplained reasons no significant difference was found among experiments ($F = 1.1$; p. $0.05 = 3.44$).

The same order of inhibition of the respiration of S-180 after treatment with 6-MP was observed in manometric experiments during which neutralized horse serum (pH 7.4) was used as a medium in place of buffered Ringer solution (mean values, each of 6 tests: $QO_2 = 7.01 \pm 0.54$ for untreated S-180; $QO_2 = 3.26 \pm 0.80$ for treated S-180).

A few experiments were undertaken in which treatment with the 7 50 mg/kg/day doses of 6-MP was not initiated until the

TABLE I. Respiration of Sarcoma 180 (S-180), Liver and Kidney (Slices).

Animals bearing tumor						Animals not bearing tumor			
Sarcoma 180		Liver		Kidney		Liver		Kidney	
U	T	U	T	U	T	U	T	U	T
6.9	3.0	—	—	—	—	—	—	—	—
6.6	2.3	—	—	—	—	—	—	—	—
6.8	2.9	—	—	—	—	—	—	—	—
7.5	2.9	—	—	—	—	—	—	—	—
6.9	2.1	—	—	—	—	—	—	—	—
6.1	1.6	—	—	—	—	—	—	—	—
*5.0	3.5	—	—	—	—	—	—	—	—
*5.4	2.7	—	—	—	—	—	—	—	—
6.0	1.3	8.7	7.3	—	—	—	—	—	—
5.9	1.1	8.6	9.1	—	—	—	—	—	—
—	—	9.6	11.9	—	—	10.9	11.1	—	—
—	—	9.4	10.4	—	—	12.5	11.1	—	—
—	—	11.2	10.7	—	—	11.5	9.9	—	—
—	—	11.7	11.7	—	—	10.4	9.7	—	—
3.8	3.1	—	—	18.2	23.0	—	—	—	—
4.0	3.2	—	—	23.3	18.8	—	—	—	—
5.2	1.7	—	—	26.5	20.3	—	—	—	—
2.8	1.8	—	—	19.7	25.7	—	—	—	—
—	—	—	—	20.7	20.8	—	—	18.7	20.0
—	—	—	—	23.4	21.1	—	—	20.4	20.5
—	—	—	—	23.7	22.1	—	—	25.5	21.9
—	—	—	—	22.8	29.0	—	—	21.7	22.9
†4.5	†1.7	—	—	—	—	—	—	—	—
†4.6	†1.0	—	—	—	—	—	—	—	—
†6.5	1.9	—	—	—	—	—	—	—	—
†6.9	2.2	—	—	—	—	—	—	—	—

T = Treated with 6-MP: 50 mg/kg/day for 7 days. U = Controls.
Values indicate O_2 consumption: mm³/mg dry wt/hr.
* During these experiments anaerobic glycolysis was also measured (Table II).
† During these experiments succinodehydrogenase activity was determined (Table III).

Analysis of variance of tumor data gave the following results: between treated and untreated F = 23.6; between experiments F = 1.0.

fourth day after implantation of the tumor. In these mice there was a reduction in the QO_2 of S-180 of the same magnitude as that described above (mean values of each of 6 tests: $QO_2 = 5.49 \pm 1.52$ for untreated tumor; $QO_2 = 2.89 \pm 1.17$ for treated tumor). By contrast treatment with a few large doses of 6-MP for a shorter period (250 mg/kg/day for 3 days starting on the 5th day after implantation) did not result in significant reduction in the respiration of S-180.

Values for the anaerobic glycolysis of tumor, liver, and kidney from treated mice are presented in Table II. The $Q^{N}_{CO_2}$ values of the treated tumors are about 35% of the control values whereas those of liver and kidney appear to have been unaltered by treatment. In a few experiments anaerobic glycolysis was determined with horse serum (pH 7.5) as the medium and the result obtained was similar to the findings in Ringer-bicarbonate (mean values, each of 6 tests, not corrected for CO_2 retention: untreated tumor, $Q^{N}_{CO_2} = 11.25 \pm 2.31$; treated tumors, $Q^{N}_{CO_2} = 4.52 \pm 1.22$).

From the above results it is evident that 6-MP induces a significant metabolic depression in S-180. It became of interest to determine whether this effect was due to a generalized lowering of all steps in cellular metabolism or whether it could result from a relatively specific biochemical lesion. For an initial survey of these possibilities the succinic dehydrogenase activities of S-180 and of liver were compared in treated and control mice. From Table III it appears that the succinic dehy-

TABLE II. Anaerobic Glycolysis of Sarcoma 180 (S-180), Liver and Kidney (Slices).

Animals bearing tumor						Animals not bearing tumor			
Sarcoma 180		Liver		Kidney		Liver		Kidney	
U	T	U	T	U	T	U	T	U	T
14.9	7.2	—	—	—	—	—	—	—	—
13.2	6.0	—	—	—	—	—	—	—	—
20.9	5.9	—	—	—	—	—	—	—	—
18.5	5.6	—	—	—	—	—	—	—	—
19.9	6.2	—	—	—	—	—	—	—	—
16.1	6.3	—	—	—	—	—	—	—	—
14.6	5.1	—	—	—	—	—	—	—	—
15.5	5.5	—	—	—	—	—	—	—	—
*27.0	9.1	—	—	—	—	—	—	—	—
*24.8	10.6	—	—	—	—	—	—	—	—
16.9	5.6	2.5	2.2	—	—	—	—	—	—
19.3	4.2	2.6	2.7	—	—	—	—	—	—
—	—	3.1	2.2	—	—	2.9	2.5	—	—
—	—	2.8	2.7	—	—	3.5	3.3	—	—
—	—	3.5	2.8	—	—	2.9	2.0	—	—
—	—	3.4	3.0	—	—	3.1	1.9	—	—
24.6	7.6	—	—	11.0	10.9	—	—	—	—
19.2	7.0	—	—	7.0	11.6	—	—	—	—
—	—	—	—	13.5	11.8	—	—	10.8	11.6
—	—	—	—	12.9	12.3	—	—	11.0	10.9
—	—	—	—	8.3	10.9	—	—	12.8	12.5
—	—	—	—	7.5	11.7	—	—	14.8	11.7

T = Treated with 6-MP: 50 mg/kg/day for 7 days. U = Controls.
Values indicate CO_2 production: mm^3/mg dry wt/hr.
* During this experiment respiration was also measured (Table I).

drogenase activity of S-180 is relatively small. It can also be observed that the treated S-180 had a lower enzymatic activity than the controls; but this difference was small and barely significant at the 5% level ($F = 5.37$; p. 0.05 = 5.32). No significant difference was found between the activity of treated and untreated liver (mean values, each of 5 tests: 17.68 ± 2.03 for untreated; 20.05 ± 3.27 for treated).

TABLE III. Succino-Dehydrogenase Activity of Sarcoma 180 Homogenates.

Untreated S-180		Treated S-180	
1.2	1.2	1.6	1.5
2.0	1.9	1.9	1.7
1.8	1.7	1.6	1.5
2.0	1.8	1.5	1.7
1.9	1.8	1.6	1.7
1.6	1.6	1.4	1.3
1.7	1.6	1.3	1.3
*1.6	1.8	1.7	1.5
*1.9	1.9	1.6	1.7
			F = 5.37

Treatment with 6-Mercaptopurine: 50 mg/kg/day for 7 days.
Values indicate O_2 consumption: mm^3/mg homogenate/hr.
* During these experiments respiration of slices of the same tumor was also measured (Table I).

Discussion. The above findings have revealed that the respiration and anaerobic glycolysis of S-180 is reduced by treatment *in vivo* with 6-MP. This metabolic change gains significance when considered in relation to the cytological alteration and inhibition of growth produced by the same treatment. It may be asked whether the observed disturbances in metabolism are the more primary effects of 6-MP that in turn result in retarded growth and altered cellular morphology. To gain further insight into this would require detailed study of various enzyme systems. Such an analysis might consider a previous suggestion that 6-MP or a ribosyl derivative might possibly become incorporated into coenzyme-like molecules in lieu of natural purines(5). The "fraudulent" coenzymes so formed could act as inhibitors of metabolism. This possibility is in keeping with recent experiments in which coenzyme A has been shown to antagonize the inhibitory effect of 6-MP in tissue cultures of S-180 and of embryo skin(4). It has also been found that the agent inhibits the acetylation of sulfonamide in rat liver homogenates (6).

Summary and conclusions. Chronic treatment with tolerated doses of 6-mercaptopurine causes a reduction of 50% or more in the respiration and anaerobic glycolysis of Sarcoma 180 while it does not impair the metabolic functions in liver and kidney. Succinic dehydrogenase activity of Sarcoma 180 is also impaired by treatment with 6-mercaptopurine, but this effect does not seem to be great enough to account fully for the observed reduction in respiration.

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An Evaluation of Thymidine in Treatment of Tropical Sprue. (22606)

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(Introduced by W. H. Crosby.)

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Spies *et al.*(1) demonstrated that thymine given orally in large doses has anti-anemic properties for pernicious anemia patients comparable to folic acid. Vilter *et al.*(2) have noted similar results with uracil. Thymine is capable of replacing folic acid in the growth of a folic acid "dependent" strain, *Lactobacillus lactis,* if vit. B_{12} is provided(3). Thymidine has been shown to be effective in replacing vit. B_{12} in the growth of a number of strains of Lactobacilli under conditions in which vit. B_{12} is ordinarily required(4,5). Thymidine also exerts a marked sparing action on the requirement for folinic acid of *Leuconostoc citrovorum*(3). These results have been taken as evidence that vit. B_{12} is involved in the linkage of desoxyribose with thymine to produce thymidine. Folic acid participates in the transfer of single-carbon units such as the steps leading to the methylation of uracil to produce thymine.

Since folic acid and vit. B_{12} are equally efficacious in the treatment of tropical sprue it seemed of interest to determine the effect of thymidine in the treatment of the megaloblastic anemia of this disease. If there is a block only in the chemical pathways leading to the formation of thymidine, the administration of this end product should correct the defect. To our knowledge there have been no published reports on the use of thymidine in sprue. It has been tried in only small amounts (5 mg) in the treatment of pernicious anemia(6).

TABLE I. Effect of Thymidine on Megaloblastic Anemia of Tropical Sprue.

Patient	Dose (mg)	Route	Total dose (mg)	Reticulocytosis
1	50	I.M.	50	None
2	50	"	50	"
3	100	"	400	"
4	200	I.V.	200	"
5	100	I.M.	600	"
6	100	"	1400	"
7	250	"	2500	"

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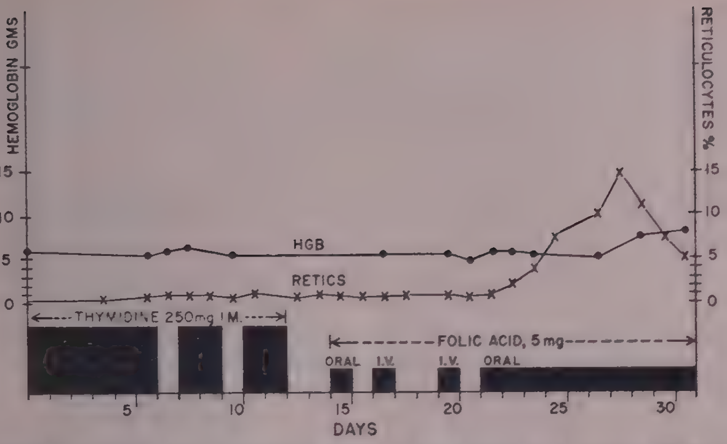

FIG. 1. Serial hemoglobin and reticulocyte values in the patient who received 2500 mg of thymidine parenterally. A reticulocytosis occurred with small amounts of folic acid given orally, but not with thymidine.

Material and methods. Seven patients with tropical sprue were studied. All gave a history of diarrhea, weakness, and weight loss. All had glossitis, malabsorption, marked anemia, and megaloblastic bone marrow. Aqueous solutions of thymidine were sterilized by autoclaving. Bacteriological assay with *L. leichmannii* after sterilization confirmed potency. Paper chromatography after sterilization gave a single spot with an Rf value identical with a non-autoclaved sample of the same material. All doses were given by intramuscular injection, except one which was given intravenously. When multiple dosage was used, injections were given daily or on alternate days. Patients were observed clinically and were followed with frequent reticulocyte counts, and hemoglobin determinations for 2 week periods.

Results. The results are summarized in Table I and Fig. 1. During the observation period none of the patients showed subjective or objective evidence of improvement. Subsequent administration of folic acid in all patients led to a reticulocytosis and rapid clinical improvement. Vitamin B_{12} is considered to be 10,000 to 30,000 times as effective in promoting growth of Lactobacilli as thymidine(7). Thus one microgram of Vit. B_{12} is equivalent to 10 to 30 mg of Thymidine. The first 4 patients possibly did not receive a sufficient dose to expect a reticulocytosis. The latter 3, however, received the equivalent of at least 20, 47 and 80 μg of Vit. B_{12} by the above criteria, and should have been expected to develop reticulocytosis of thymidine deficit were the sole metabolic defect.

Summary. Injections of a potent preparation of Thymidine in doses equivalent to at least 20, 47, and 80 μg of Vit. B_{12} were without effect in producing hematologic improvement in patients with tropical sprue in relapse.

The thymidine used in this study was purchased from Nutritional Biochemicals Corporation.

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Concentration of Dilute Virus Suspensions by Alum Flocculation. (22607)

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(Introduced by Isaac Ruchman.)
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Most chemical and physical methods for virus concentration and/or purification have been developed for treatment of potent rather than weak virus suspensions. Melnick's(1) procedures for detection of poliomyelitis and Coxsackie viruses in sewage are expensive and complicated. In this laboratory, treatment by Kelly's(2) ion-exchange resin absorption technic of waters lightly contaminated with Coxsackie virus failed to yield virus.

A better solution to the problem of concentrating dilute virus suspensions was suggested by an observation that the floc complex formed in the aluminum sulfate flocculation of artificially contaminated water contained in excess of 99% of added Coxsackie virus and that significant amounts of virus could be recovered from the floc. This observation confirmed the findings of Chang, *et al.*(3), who recovered bacterial virus from the floc complex.

Aluminum hydroxide, either as a preformed gel or as a coprecipitate, has been used for adsorption of various toxins, bacteria and viruses. Schaeffer and Brebner(4) and Sabin (5) used aluminum hydroxide gel for adsorption of poliomyelitis virus from tissue suspensions and they determined pH optima for adsorption and elution. Although no quantitative results were reported in their papers, our experience with preformed floc or aluminum hydroxide gel indicates that recovery by this method is not high.

This communication reports a sensitive and effective, yet convenient and inexpensive method for concentration of Coxsackie virus from dilute suspensions.

Materials and methods. Virus. Group A, type 2 Coxsackie virus of the 16th or 17th mouse passage was used as the source of virus. A 20% suspension of infected muscle, centrifuged at 2,000 rpm for 30 minutes to remove large tissue particles, served as stock virus and was diluted in 1% peptone (Difco) for use. The stocks titered 1 X $10^6 LD_{50}$/0.02 ml and 0.89 X $10^6 LD_{50}$/0.02 ml. These values were determined from titrations with at least 200 mice for each assay.

To serve as an additional check on virus titers, a dilution tube containing an estimated 1.0 or 0.5 LD_{50} was used for animal inoculation and for preparation of successive dilutions. Thus, each series of dilutions employed in seeding the experimental mixtures was represented by a measurable control. Since the theoretical dosage in the initial mixture before flocculation could not be measured except by inoculation of 100 or more mice, yields of virus were then compared to the measurable control titer in order to estimate percentage recovery.

Reagents and Glassware Cleaning Procedures. 1. All glassware which came into contact with virus was soaked overnight in a solution of Hemo-Sol (Meinicke Co., N.Y.) in order to remove its virus adsorbing capacity [Chang(3)], rinsed 5 times in cold tap water, and once in distilled water before sterilization. 2. The initial pH of the flocculation mixtures (pH 6.2-6.5) was controlled by the ratio of $NaHCO_3$, to $Al_2(SO_4)_3 \cdot 18H_2O$ and was adjusted by means of pH determinations on virus-free controls. 3. Reagents: $NaHCO_3$, 0.1 M; $Al_2(SO_4)_3 \cdot 18H_2O$, 1% solution; Tryptose Broth (Difco), 2% solution; SiO_2 (Activated Silica, 200 mesh, Davison Chemical Corp.), 1% suspension. Reagents were sterilized by autoclaving at 15 lb for 15 minutes except solutions containing sodium bicarbonate. The latter were sterilized by filtration through an 0-3 Selas filter. $NaHCO_3$, 0.1 M; Na_2CO_3, 0.1 M; and HCl, 0.1 N were used to formulate the buffers used for recovery of virus from the floc. The pH values of the solutions were checked with a glass electrode pH meter both before and after filtration.

Flocculation procedures. Into a 600 ml beaker, 370.8 ml of sterile distilled water, 0.4 ml of SiO_2 suspension, 10 ml of tryptose broth, 10 ml of $NaHCO_3$, and 1.0 ml of diluted virus were added in order. A sterile glass paddle-type stirrer was then placed inside the beaker, the assembly connected to a stirring apparatus and after stirring at 81 rpm for 2 minutes, 7.8 ml of $Al_2(SO_4)_3 \cdot 18H_2O$ (7.8 ml for 100 parts per million) was added, and stirring resumed. Floc formation started within one or 2 minutes and continued with progressive increase in particle size. After 30 minutes of stirring, the stirring rod was removed from the beaker and the floc allowed to settle for 60 to 90 minutes. Speed of stirring proved to be critical. Higher speeds tended to break up floc particles and made settling very slow. After settling occurred, the supernatant fluid was drawn off by means of suction, care being taken not to disturb the floc. The floc was then transferred to a 10 ml mixing cylinder* adapted to a 100 ml centrifuge shield and centrifuged at 1,500 rpm for 5 minutes, to pack the floc. After the removal of the supernatant fluid, the floc was resuspended in a buffer of appropriate pH to a volume of 10 ml.

After standing 1 to 3 hours at room temperature with intermittent agitation, the floc was again centrifuged at 1,500 rpm for 5 minutes and the supernate collected and used for animal inoculation.† One to 3-day-old suckling mice were inoculated subcutaneously with sample material in 0.02 ml quantities and were observed for signs of Coxsackie virus infection for at least 10 days. Methods employing ion-exchange resin were those described by Kelly(2) and were included for a comparison of the two methods.

Results. Using the same virus inoculum, the ion-exchange method was compared to the flocculation method, using 60, 80, and 100 ppm and one and 3 hour redispersing times in the latter. The flocculation method resulted in higher recoveries of virus in all combinations tested (Table I).

TABLE I. Comparison of Alum Flocculation and Ion-Exchange Resin Concentration.

Temp., 26-28°C; pH of water after flocculation, 6.3; pH of buffer used for separation, 8.0; estimated initial virus dosage, .025 LD_{50}/.02 ml; estimated final virus dosage, 1.0 LD_{50}/.02 ml (40 × conc.).

Exp.	Conc. of aluminum sulfate, ppm	Floc redispersing time, hr	Mice, infected /total	% mortality
1	60	1	21/43	48.8
		3	13/38	34.2
	80	1	26/54	48.1
		3	8/22	36.3
	100	1	28/53	52.8
		3	13/28	46.4
	Control at estimated 1 LD_{50} per .02 ml		13/20	65.0
2	60	1	5/14	35.7
		3	3/17	17.6
	80	1	7/40	17.5
		3	11/40	27.5
	100	1	7/33	21.2
		3	17/45	37.7
	Ion-exchange-resin method (40× conc.)		2/33	6.1
	Control at estimated 1 LD_{50} per .02 ml		20/46	43.5

The flocculation results in Table I were examined by a series of statistical significance tests. Since Exp. 2 showed lower percent mortality than Exp. 1, the data for these 2 tests were handled separately, except in one case described below.

In all cases the following chi-square (χ^2) test was applied:

$$\chi^2 = \frac{\sum \frac{x_i^2}{n_i} - \frac{(\Sigma x_i)^2}{\Sigma n_i}}{\hat{p}\,(1-\hat{p})}$$

with degrees of freedom equal to one less than the number of percentages compared, where x_i denotes the number of mice killed out of n_i inoculated, and $\hat{p} = \Sigma x_i / \Sigma n_i$ denotes the average proportion response over the conditions being compared. [See Cochran(6)]. Responses were compared under each of the

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* Kontes Glass Co., Vineland, N. J.

† Volume relationships in the procedure outlined resulted in a 40 fold concentration and were selected for convenience. Other volumes may be selected so that concentrations of 100 fold or greater may be obtained. For practical use, however, concentrations of greater than 200 fold are difficult because of the floc volume and the molarity of the buffer.

TABLE II. Effect of pH of Floc-Redispersing Fluid on Recovery of Coxsackie Virus from Water Concentrated 40 Times by Alum Flocculation Method.

Temp., 26-28°C; pH of water after flocculation, 6.3; dosage of aluminum sulfate, 100 ppm; estimated initial dose of virus in water, .025 LD_{50}/.02 ml; estimated amount of virus after redispersing, 1.0 LD_{50}/.02 ml.

pH of redispersing fluid*	Redispersing time, hr	Mice, infected /total	% mortality
7.5	1	1/11	9.1
	3	1/20	5.0
7.9	1	12/34	35.3
	3	9/34	26.4
8.5	1	12/37	32.4
	3	(4/7)†	
8.9	1	12/37	32.4
	3	8/23	34.7
Control made without alum at an estimated dose of .5 LD_{50}/.02 ml		15/52‡ 12/47§	28.8 25.5

* Bicarbonate buffer was used for pH 7.5 and carbonate buffer was used for the 3 higher pH values.

† No. of mice used was too small because of cannibalism in 3 cages.

‡ Control made after 1 hr standing.

§ " " " 3 " " .

following sets of conditions for each test number: 1. Different concentrations of alum within each redispersing time. 2. Redispersing times, averaging percent mortality over the 3 alum concentrations. 3. Alum concentrations, averaging over the 2 redispersing times. 4. All experimental conditions combined *vs.* control.

Finally, for the 3-hour redispersing time, average response after 60 and 80 ppm alum flocculation in both experiments was compared with the average response after 100 ppm alum.

The only clearly significant results were those comparing the over-all experimental averages with the controls. There was some indication, though not statistically significant, that 100 ppm alum gave somewhat better percent recovery of potency (mortality) than the other 2 concentrations. Therefore, the 1-hour redispersing time and 100 ppm alum were chosen for future experiments on a practical basis. On the average, sufficient virus was recovered to achieve about 70% of the potency (mortality) exhibited by the control.

Table II presents the results of an experiment designed to select the optimum pH for recovery of virus from flocs formed with 100 ppm of aluminum sulfate. $NaHCO_3$-HCl buffer at pH 7.5 was clearly not effective in separating virus. Analysis of the data on the remaining buffers by the chi-square test revealed no significant variation among percentage responses after separation at pH values of 7.9, 8.5, or 8.9 with 1-hour or 3-hour redispersing times. On the average, percentage mortality among mice inoculated with virus recovered from flocculation was 18% higher than that shown by the control material which had an estimated dosage of only 0.5 LD_{50} per 0.02 ml.

A precise estimation of percentage virus recovery cannot be given for these results unless a dosage-mortality curve is invoked, since percentage mortality is not always directly proportional to dilutions used. It may be stated, however, that a potency in excess of 0.5 LD_{50} but less than the theoretical 1 LD_{50} was recovered. This indicates, on a volume basis, that greater than 50% of potency was recovered.

The results of additional experimentation with the ion-exchange method are presented in Table III. Since the volume of resin required by this method is so large that it is difficult to collect the final supernate without contaminating it with resin, the concentration was reduced to 20 times so that twice the amount of supernate would be available. In these replicates the virus dosage in the beakers was doubled so that the final virus titer would be comparable with the levels in the 40-fold concentration.

When the percentage mortalities of these replicates are compared with the results ob-

TABLE III. Replicate Experiments with Ion-Exchange-Resin (Dowex I) Concentration Method.

Estimated initial virus titer, .05 LD_{50}/.02 ml
" final " " , 1.0 "

Mice, infected/total	% mortality
1/32	3.1
2/35	5.7
1/27	3.7
2/21	9.5

TABLE IV. Recovery of Coxsackie Virus from Water Concentrated 40 Times by Alum Flocculation Method.

Temp., 26-28°C; pH of water after flocculation, 6.3; dosage of aluminum sulfate, 100 ppm; pH of floc redispersing fluid, 7.9; estimated initial dose of virus in water, .00625-.025 LD_{50}/.02 ml; estimated amount of virus after redispersing, .25-1.0 LD_{50}/.02 ml; Redispersing time, 1 hr.

Estimated amt of virus per .02 ml of concentrate	Test No.	Mice, infected /total	% mortality
1 LD_{50}	1	16/80	20.0
	2	21/50	42.0
.5 "	1	10/51	19.6
	2	10/37	27.0
.25 "	1	2/100	2.0
	2	5/32	15.6
Controls made without alum at an estimated (Test 1) .625 LD_{50}/.02 ml (Test 2) 1.0 LD_{50}/.02 ml	1	12/47	25.5
	2	10/18	55.5

tained in the flocculation experiments in Tables I and II, it is obvious that the flocculation method is superior to the ion-exchange method in recovering virus activity.

Table IV shows the results obtained when water contaminated with lower amounts of virus was treated by flocculation. It will be noted that water containing as little as 0.00625 LD_{50}/0.02 ml can be flocculated, and that sufficient virus can be recovered to induce infection in 2 to 15% of the inoculated mice.

Discussion. This development of a sensitive and economical method of virus concentration fulfills a need which has hampered investigators in virus fields. Coxsackie virus was employed in these investigations because of its obvious sanitary significance as an enteric virus. Preliminary work on APC type 3 virus, not detailed here, has shown that virus also to be amenable to this concentration procedure, and it is possible that application of the flocculation method is feasible for detection of residual live virus in vaccines such as are now being produced for poliomyelitis immunization. Chang, *et al.*(3) reported much longer times were necessary for recovery of bacterial virus from the floc complex than we observed for Coxsackie virus. This may well represent inherent differences between the types of virus used in their work and the Coxsackie virus used in this work. Further investigation of these differences is in progress, particularly with respect to virus purification procedures.

Summary. A method for the concentration of Coxsackie virus from dilute suspension is described. This flocculation procedure is capable of detecting as little as 0.00625 LD_{50}/0.02 ml in the starting material and is shown to be more sensitive than an ion-exchange method currently used. It possesses the additional advantages of being economical and simple in execution.

We are indebted to Dr. E. K. Harris for the statistical analyses contained in this paper and to Mr. Richard A. Gallagher for competent technical assistance.

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Direct Complement-fixation Test for Diagnosis of Ornithosis in Turkeys.* (22608)

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The presence of ornithosis antibodies in turkeys has been known since 1942(1). The importance of turkeys as a source of human infection was reemphasized in 1948, and thereafter, by outbreaks of psittacosis among employees of turkey dressing plants(2,3) and by the subsequent isolation of a highly toxigenic ornithosis turkey strain(4). More recent studies have revealed the widespread occurrence of the virus in turkeys, and have led to the recognition of epizootics of varying degrees of severity which have been responsible for human infections. In view of the foregoing, rapid and sensitive methods for the detection of this disease in fowls are required.

The complement-fixation (CF) test has not been applied in the study and diagnosis of turkey ornithosis since, among other avian sera, Rice(5) reported the failure of antipullorum turkey sera to fix complement with *S. pullorum*, and Eddie and Francis(1) observed fixation at only low dilutions with a limited number of turkey antiornithosis sera. The presence of a specific inhibitor, presumably an incomplete type antibody, was demonstrated in avian non-complement fixing sera (5,6), and thus the indirect complement-fixation test (ICF) was devised for the detection of such antibodies(5,6). Hilleman and Helmold(7) suggested the use of the ICF for fowl antiornithosis sera, and Karrer, *et al.*(8) studied the variables involved in this test and applied the method for epidemiological surveys of ornithosis in chickens, ducks and turkeys(9). Since that time, the ICF has been used almost exclusively for the routine serological testing of turkey sera.

A previous communication from this laboratory described the extraction and characterization of soluble CF and skin-test antigens from the psittacosis virus(10). The CF antigen was found to fix complement consistently in the presence of turkey antiornithosis sera. Considering the ease of performing the direct complement-fixation test (DCF) when compared to the indirect method, an evaluation of the DCF was undertaken.

Materials and methods. The method of preparation of the detergent-extracted antigen was described previously(10). Meningopneumonitis virus (Francis) was used exclusively in the present study. To maintain maximum CF activity, the crude extract was cleared by relatively slow speed centrifugation (10,000 x G for 20 minutes), and used without further purification. The detergent extract could not be used for the ICF with sera giving positive DCFs, since direct fixation interfered with the inhibition reaction. Therefore for the ICF, these sera were tested with either a concentrated heated phenolized meningopneumonitis allantoic fluid suspension or a psittacosis (stain 6bc) yolk-sac particle suspension.

Sera. Groups of turkey sera were obtained from the following sources: A) year-old Broadbreasted Bronze turkeys from Oregon which had experienced a severe ornithosis epizootic approximately 3 months prior to bleeding;† B) 2- to 5-year-old Broadbreasted Bronze turkeys from the same ranch as flock A, but in which ornithosis was not evident;† C) a flock from the same vicinity as A, but without evidence of ornithosis infection;† D) sera from birds that had undergone a natural infection 18 months prior to bleeding;‡ E)

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† These turkeys were generously made available to us by Dr. Don Mason, U. S. Public Health Service, and Dr. Monroe Holmes, Oregon State Board of Health.

‡ Kindly supplied by Dr. Raymond Bankowski, School of Veterinary Medicine, University of California, Davis.

TABLE I. Comparison of Direct and Indirect Complement-Fixation Tests for Detection of Turkey Ornithosis.

Group	History of infection	No. turkeys tested	DCF* pos ICF† ”	DCF pos ICF neg	DCF neg ICF pos	DCF neg ICF ”	% positive DCF	% positive ICF
A	Natural (3 mo)	47	41	3	0	3	93.6	87.3
B	No evidence	61	1	1	0	59	3.2	1.6
C	” ”	50	2	0	0	48	4.0	4.0
D	Natural (18 mo before)	12	7	5	0	0	100	41.6
E	Exp. infected 8 wk	11	7	0	2	2	63.7	81.8
	Total	181	58	9	2	112	36.0	33.1

* Direct complement-fixation. † Indirect complement-fixation.

one-year-old Beltsville white turkeys experimentally infected with ornithosis virus (strain J.O.) by the intratracheal route 8 weeks before testing.§

Serological methods. The ICF was performed similarly to the method described by Karrer, *et al.*(8). For the DCF, 4 units of the detergent-extracted antigen are used. The preliminary incubation in both tests was carried out at 37°C for 75 minutes, and after the addition of sensitized sheep erythrocytes, a secondary incubation at 37°C for 30 minutes was made. Titers represent the reciprocal of the highest dilution of serum showing either a 1+ or 2+ reaction in the ICF (partial inhibition) or a 4+ reaction (complete fixation) in the DCF.

Results. When it was determined that the crude detergent extract would fix complement with randomly selected turkey antiornithosis sera, a survey of naturally infected and experimentally infected turkeys was made using the DCF. In some cases the ICF and DCF were compared. Forty-seven turkeys were selected at random from a flock that had undergone a severe epizootic several months prior to bleeding. Table I shows that 41 sera in this group (A) were positive by both the ICF and DCF, and that 3 sera were positive in low dilutions (1:4, 1:4, 1:16) in the DCF and negative in the ICF. Dr. B. Eddie of The George Williams Hooper Foundation, University of California Medical Center, San Francisco, kindly tested these sera by the DCF, and they were all negative to 4 units of a heat-killed psittacosis (strain 6bc) yolk-sac antigen. The significance of this observation will be discussed later. Also shown in Table I are results with sera from 2 flocks (B,C) in which there was no evidence of infection at the time of bleeding. The large number of negative sera attest to the specificity of the DCF. One serum from flock B was negative by the ICF and had a DCF titer of 1:4. To determine the duration of the DCF antibody, sera were obtained from 12 turkeys which had undergone a natural infection 18 months prior to the test. Only 5 sera had positive ICF titers; whereas, all sera had antibodies as revealed by the DCF (Table I).

Table II presents DCF titers of 20 turkeys experimentally infected for 8 weeks, and a comparison of the DCF and ICF titers of 11 of these sera. Two of the negative DCF sera were positive in the ICF. These findings sug-

TABLE II. Complement-Fixation Reactions of Turkeys Experimentally Infected 8 Weeks with Ornithosis.

Turkey	Titers Direct test	Titers Indirect test
9	8	32
18	4	8
46	16	8
63	32	32
64	256	16
85	0*	0
89	0	4
105	32	8
106	32	16
117	0	8
136	0	2
10	128	—
26	2	—
48	32	—
77	64	—
101	256	—
116	128	—
123	64	—
125	32	—
141	32	—

* Negative at 1:2 dilution.
— = Not tested.

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§ Kindly supplied by Dr. Don Davis, A. and M. College of Texas, College Station.

TABLE III. Appearance of Complement-Fixing Antibodies in Experimentally Infected Turkeys.

	Days after infection		
Turkey	0	8	14
1	0*	2 (3+)	64
2	0	16	32
3	0	2 (3+)	8
4	0	2	64
5	0	4	8

* Negative at 1:2 dilution.

gested that if incomplete CF antibodies were formed in turkey sera, they might have been predominant during the early stages of the infection. However, on the contrary, positive DCFs were demonstrated 1-2 weeks following infection. Five one-year-old Beltsville white hens were infected with over 10^7 $LD_{50}s$ (yolk-sac titration) of psittacosis virus (Texas turkey strain). All turkeys were negative prior to infection. Eight days following infection all turkeys showed DCF titers, and the titers increased by 14 days of infection (Table III). Thus, the earliest appearance of the DCF antibodies paralleled the reported appearance of the ICF antibodies in chickens (9,11).

To determine the relationship between the titers obtained by the 2 methods, a direct correlation analysis on the basis of the log distribution of the ICF and DCF titers of flock A was made.‖ Fig. 1 shows the regression line obtained, and the correlation was found to be 0.72, which was significant at the 1% level. Although there is no direct evidence as yet that the same antibody was being measured by the 2 tests, the DCF titers correlated well with the ICF titers of these sera. Prozones rarely occurred and then only in the 1:2 dilutions. Of the 91 positive sera examined to date, only one manifested an extended prozone. Attempts to eliminate the prozone of this one serum by extending the fixation period at 5°C failed. Employing the detergent-extracted antigen, attempts to obtain direct fixation of complement with chicken antisera met with repeated failures. Under the test conditions, chicken antibody truly represented the type that fails to fix complement, although chicken antiornithosis sera will precipitate with suitable antigens(12).

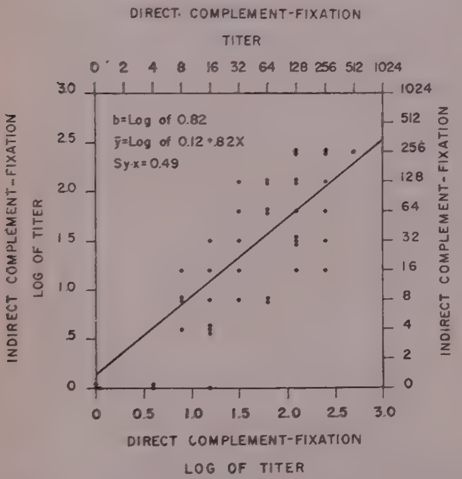

FIG. 1. Correlation analysis of direct and indirect complement-fixation reactions with turkey antiornithosis antibody.

In the light of previous claims that turkey sera fixed complement poorly(5,8), the consistency and sensitivity of the DCF in this study was surprising. The results suggested that successful fixation depended, in part, on the type of antigen used in the test. To test this hypothesis, a comparison was made of the detergent extract and other antigens commonly used in the DCF. A heated phenolized psittacosis (strain 6bc) yolk-sac antigen, a viable concentrated meningopneumonitis virus suspension (obtained by sedimentation of the virus from allantoic fluid), and the detergent extract were each titrated against 4 units each of rabbit and turkey antipsittacosis sera. The results summarized in Table IV reveal that the soluble antigen had the same titer when tested against rabbit and turkey sera. On the other hand, the allantoic fluid and yolk-sac antigens were considerably less active when titrated with turkey than with rabbit antiserum. The possibility existed that the detergent extract consisted of an antigen that differed either quantitatively or qualitatively from that found in the particle suspensions. Centrifugation of the soluble antigen at 35,000 x G for 30 minutes was known to sediment a CF antigenic fraction(10); thus, the

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‖ The authors gratefully acknowledge the efforts of Miss Peggy Stimmel of this Department for the statistical analysis.

TABLE IV. Complement-Fixing Activity of Detergent Extract and Viral Suspensions when Tested with Rabbit and Turkey Antipsittacosis Sera.

Antigen	Psittacosis antiserum Rabbit*	Psittacosis antiserum Turkey*
Crude detergent extract		
AP	32†	32
AQ	64	64
Crude detergent extract ultracentrifuged		
Supernate	16	16
Pellet	8	8
Living meningopneumonitis allantoic fluid suspension	128	16
Killed psittacosis yolk-sac suspension	256	8

* 4 units of antiserum. † CF titer of antigens.

pellet and supernatant fractions obtained by this treatment were each titrated against rabbit and turkey antisera. Table IV shows that activity of the pellet antigen equals activity of the supernate antigen with rabbit or turkey antiserum.

Discussion. Employing the yolk-sac cocto antigen (6bc), Karrer *et al.*(9) demonstrated inhibition titers with a small number of pigeon sera which gave negative results in the DCF. These investigators stated that the inhibiting and DCF antibodies rarely occur in the same serum, and that this was evidence that the inhibiting and complement-fixing antibodies were different. Our studies show, however, that when using the detergent-extracted antigen, the DCF was as accurate as the ICF for the detection of ornithosis antibody in the turkey sera studied, and that with some sera the DCF was a more sensitive indicator than the ICF. Furthermore, all but a few positive sera gave both positive indirect and direct CF reactions. These conflicting observations can be explained on the basis of the types of antigens employed. Direct fixation with turkey sera would be missed if the virus suspension antigens were standardized with non-avian or pigeon sera and then the usual 4 units of antigen were used in the direct test with turkey sera. When tested with rabbit antiserum, 4 units of the allantoic fluid and yolk-sac antigens shown in Table IV represent titers of 32 and 64, respectively; concentrations that were insufficient to fix complement with turkey sera. At least for turkey antisera, these data suggest that the inhibiting and complement-fixing antibodies may represent the same antibody, and that their detection partially depends upon the molecular configuration of the antigen. The ability of the detergent extract to fix complement with turkey antibody may be due to the physical state of the antigen. The reactive sites of the soluble antigen may be more readily available, and the union with antibody and complement is probably firmer than that found with the virus preparations. The failure to detect serious prozone effects, the early appearance in infection of the DCF antibodies, and the detection of antibodies in turkeys infected 18 months previously strengthen the practical use of the DCF.

Summary. Employing a soluble antigen, complement-fixation reactions were obtained with turkey antiornithosis sera. Antigens consisting of viable allantoic and killed yolk-sac virus suspensions did not show the same degree of activity. The direct complement-fixation reaction was as sensitive as the indirect complement-fixation test; therefore, the use of the direct test for the study and diagnosis of turkey ornithosis was suggested.

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Catalase Activity and Ethanol Metabolism in the Rat.* (22609)

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Until recently the sole function of catalase in living cells was thought to be that of preventing accumulation of toxic concentrations of hydrogen peroxide. Keilin and Hartree (1,2), however, indicated that catalase may catalyze a peroxidatic oxidation of ethanol to aldehyde. If this reaction does occur *in vivo,* ethanol oxidation conceivably may be carried out by both catalase and alcohol dehydrogenase, and not by the latter alone. Jacobsen (3) has even postulated that catalase is responsible in man for oxidation of 20% of a given dose of ethanol. Based on a review of the literature, and on his own work, Bartlett (4) concluded that alcohol dehydrogenase alone is responsible for the first step in the oxidation of ethanol. Westerfeld(5) agreed in principle with Bartlett when the former expressed the view that the catalase mechanism is not of major importance in alcohol metabolism. One of us(6) has recently attempted to determine the importance of catalase in alcohol metabolism in dogs by correlating rate of ethanol metabolism and hepatic catalase activity in the same animal. The coefficient of correlation between the two determinations was statistically significant, 0.602. Liver weight alone, however, when correlated with rate of metabolism yielded a coefficient of 0.797. In view of these findings, it appears that liver weight may be the determining factor in the calculations, and the relatively high value of the coefficient is merely due to the preponderant effect of liver weight. In view of these considerations, and because a new research technic has become available, a different approach was made to the problem of ascertaining the importance of catalase activity in ethanol metabolism.

The new approach was made possible when Heim *et al.*(7) injected rats intraperitoneally with 3-amino-1, 2, 4-triazole (designated hereafter as AT) and found that hepatic catalase activity was markedly depressed for at least 12 hours. The general plan of the present investigation involved determination of the rate of ethanol metabolism in rats, over a 4 hour period after AT had been injected, followed by determination of the ethanol oxidizing capacity of the liver, believed to be a measure of alcohol dehydrogenase activity, and hepatic catalase activity. The findings in control animals were compared with those in AT-injected experimental animals.

Methods. Twenty per cent ethanol, in saline, in dosages of 3 g/kg, was administered intraperitoneally in adult male Sprague-Dawley rats, 60 minutes after 1 g/kg of AT,‡ as a 14% aqueous solution, had been administered intraperitoneally. Control animals were given alcohol in an identical manner but were not injected with AT. Four hours after administration of ethanol, the rat was sacrificed by a blow on the head. A small specimen of liver was removed, weighed, and homogenized. Alcohol dehydrogenase and catalase activities were determined by methods previously described(6). The carcass was comminuted and treated as described by Aull *et al.*(8). To ascertain the degree and persistence of the reduction of hepatic catalase activity, as a result of the action of AT, the catalase activity was determined in groups of 3-4 rats each at hourly intervals over a 5-hour period. The assay method employed(6) was the same as that used with the small specimen in the larger experimental series.

Results. The rate of ethanol metabolism, in mg/kg/hr, and the hepatic alcohol dehydrogenase in mg/g liver/hr, and catalase activities, in m.e./mg liver and m.e./mg N, are

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‡ Contributed by the American Cyanamid Co., Agricultural Chemical Division.

TABLE I. Rate of Ethanol Metabolism and Enzyme Activities.

	Metabolic rate, mg/kg/hr	Oxid. cap., mg/g/hr	Catalase m.e./mg liver	Catalase m.e./mg N
Controls (10 rats):				
Range	180 – 239	4.5 – 9.5	.992 – 1.448	24.9 – 34.7
Mean ± S.D.	205 ± 18	7.2 ± 1.3	1.170 ± .147	29.1 ± 3.8
Exp. (10 rats):				
Range	143 – 230	6.5 – 12.0	.268 – .372	6.9 – 10.0
Mean ± S.D.	193 ± 27	8.9 ± 1.6	.327 ± .035	8.2 ± 1.0

recorded in Table I. In the rats injected with AT, determination of the liver catalase activity, at hourly intervals for 4 hours, in 3 animals each, showed a reduction of 88.1-81.7% below the average for the control series. In light of the greater depression during the first 4 hours, the catalase activities recorded in Table I probably represent maximum levels of activity reached during the 5 hour period following AT injection. The differences between mean values of both the metabolic rate and the ethanol oxidizing capacity of the liver, in the control and experimental series, were statistically tested. In each case the P value, of less than 0.1, indicates that there is probably no significant difference between control and experimental series.

Discussion. Results of the determinations of hepatic catalase activity over a period of 5 hours, indicate that AT depresses catalase activity promptly and that the depression persists to a marked degree for at least 5 hours. This marked reduction in catalase activity of the liver, averaging at least 71.8%, should be sufficient to affect the rate of ethanol metabolism if catalase plays an important role in alcohol metabolism. The p values, indicating that both the rate of ethanol metabolism and the ethanol oxidizing capacity of the liver are statistically identical in control and experimental series, lead us to conclude that hepatic catalase does not play a significant role in alcohol metabolism. Apparently, AT does not have an appreciable effect upon alcohol oxidizing capacity of the liver. This determination, originally designated as ethanol oxidizing capacity of liver(6) is now believed to be a measure of the alcohol dehydrogenase activity since addition of purified catalase, or of purified catalase plus perborate, to the liver homogenate failed to give any increase in the ethanol oxidizing capacity of liver homogenate. All of the observations, therefore, indicate that liver catalase does not play a significant role in ethanol metabolism.

Summary. Reduction in hepatic catalase activity, of at least 71.8%, over a period of 5 hours, as a result of injection of 3-amino-1, 2, 4-triazole, failed to produce a statistically significant alteration in rate of ethanol metabolism in rats. The ethanol oxidizing capacity of the liver, *e.g.*, alcohol dehydrogenase activity, was statistically the same in control and experimental series. It is concluded that hepatic catalase, in rats, does not play a significant role in ethanol metabolism.

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Identification of Avian Erythroblastosis Virus by Precipitation with Chicken Immune Serum.* (22610)

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Particles characteristic of avian erythroblastic leukemia(1) have been observed(2) in electron micrographs of the plasma of chicks with the disease. Morphologically, the particles were similar in shape and size to those occurring under like conditions in myeloblastosis, which is another form of avian leukemia. In the latter instance, the particles have been identified(3,4) as the viral agent of the disease. The findings already described (2) have suggested that the particles found in erythroblastosis might represent the etiologic agent. One means for establishing the identity of the virus of myeloblastosis consisted in the electron micrographic study(4) of the precipitin reaction with specific antiserums from the chicken. A similar technic has been applied in erythroblastosis. Chickens repeatedly injected with formalin-treated particles concentrated by ultracentrifugation of large volumes of plasma, develop antibodies strongly neutralizing the infectivity of the virus. Electron micrographic examination of mixtures of the antiserums with particle concentrates revealed specific clumping of the characteristic particles. The results of the precipitin studies, which establish the serological identification of the particles with the virus of erythroblastic leukosis, are here described.

Materials and methods. The disease under study is an apparently pure strain of erythroblastosis obtained(5) from Dr. Astrid Fagraeus (State Bact. Lab., Stockholm), but derived initially in the laboratory of Dr. J. Engelbreth-Holm (Dept. of Pathol. Anatomy, University of Copenhagen). It has been passed routinely in this laboratory in White Leghorn chicks and chickens by intravenous inoculations of 0.1-ml volumes of *filtered* plasma or dilutions of it. The birds were always White Leghorns of Line 15 inbred at the Regional Poultry Research Lab., East Lansing, Mich., for high susceptibility to lymphomatosis(6). The characteristics of the disease have not changed since the earlier description(5).

Particle concentrates for preparation of immunizing antigen were obtained by ultracentrifugation of plasma from diseased birds. Electron micrographic examination of many plasmas has shown that the particle content in erythroblastosis reaches a maximum of approximately 2×10^{10} per ml, in striking contrast with myeloblastosis in which particle counts reach levels of 2×10^{12} virus particles per ml. There is wide variation in the particle content in erythroblastosis, some of the plasmas containing practically none demonstrable by physical methods. Moreover, as already indicated(5), there was no means for selecting the best plasmas since the adenosine triphosphatase micro screening test(7) useful in myeloblastosis was not applicable in erythroblastosis. For these reasons, donors of the plasmas had to be chosen at random, and because of the low average particle content, large volumes of plasma were necessary to provide the mass of particles sufficient to serve as effective antigen. Fortunately, older birds are more susceptible(5,8) to this virus than the baby chicks required for myeloblastosis (9), and consequently, volumes of plasma adequate for this purpose were readily obtained. Birds of 2 to 20 weeks of age with the disease were bled from the heart shortly before death was expected. Heparin was used as anticoagulant. Cells and large particles were removed by centrifugation once at 850 x g for 15 minutes, and a second time at 2700 x g for 20 minutes. The supernatant fluids were then spun at 20,000 x g for 60 minutes

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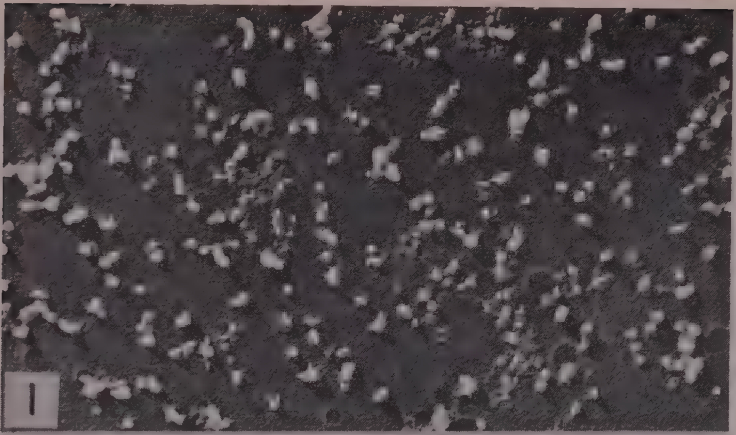
to sediment the particles which were resuspended in Ca-free Ringer's solution at about 25 x concentration. Formalin in Ca-free Ringer's solution was added in amounts sufficient to bring the suspensions to 10-20 times concentration of particles with respect to the original volumes of plasma and 0.1% concentration of formalin. The suspensions were stored in the refrigerator for about 3 weeks. The vaccine was prepared in batches, at intervals, from a total of 2,125 ml of plasma derived from 198 chickens. This whole amount of vaccine was used for immunization of 8 roosters (Line 15 White Leghorns) of 6.5 months age at the time of the first injection. Each was given 1.0 ml vaccine intraperitoneally twice on alternate days. After 1 week, a series of 3 injections of 2.0 ml was made on each of alternate days. The third course of 2.0, 2.5 and 2.5 ml was given in the same way after an ensuing week. There was no evidence of disease in the vaccinated birds. All were bled from the heart 11 days after the last injection, and the serum was stored at —22°C.

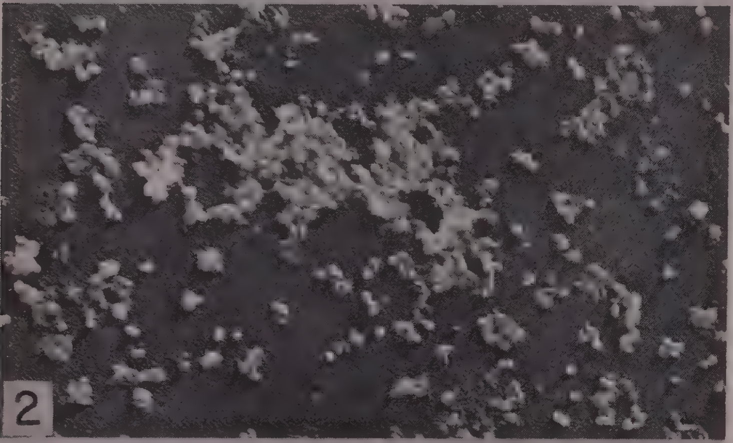
Precipitin experiments were carried out with particle concentrates similar to those used for the immunizing antigen. After removal of the cells by centrifugation, the plasmas were stored at —78°C until used, then thawed in running tap water and chilled in an ice bath. Preparation of the particle concentrates was carried out at 2-5°C. In a typical preparation, 4 plasmas, 101 ml, were centrifuged 20 minutes at 2,100 x g, and the supernatant fluids spun 30 minutes at 29,000 x g in the Servall SS-1 rotor to sediment the particles. The small, yellowish clear pellets were resuspended and combined in a total volume of 12 ml of 0.14 NaCl containing 0.001 M sodium phosphate, pH 7.0. The cloudy suspension was stirred 2 hours and then centrifuged 20 minutes at 2,500 x g. The supernatant fluid was decanted from the white opaque pellet and centrifuged 30 minutes at 20,000 x g in an airdriven vacuum chamber centrifuge. The supernatant fluid was decanted and the pellet resuspended in 1.0 ml sodium chloride phosphate solution as before. The pellet suspension was stirred overnight and then clarified by centrifugation at 2,500 x g for 5 and then for 15 minutes.

The immune serum and the serum from a normal (uninfected) bird from the same flock were heated 30 minutes at 56°C to inactivate complement; after cooling, the serums were centrifuged 30 minutes at 47,000 x g in a small horizontal cell to eliminate particulate material, and the upper two-thirds of the supernatant taken for use. For the precipitin reaction, 1 part particle concentrate, 1 part serum and 1 part NaCl solution were mixed so that the final concentration of sodium chloride was 7.5%(10). Whether the particle concentrate and serum were first dialysed against 7.5% NaCl or simply mixed with more concentrated NaCl solution appeared not to affect the results. The mixtures were kept at 37°C for 2 hours, and then at 2°C. Samples were removed at intervals and diluted 333 fold with 0.9, 4 or 8% sodium chloride solution for electron micrography. The particles in these dilute samples were then deposited by ultracentrifugation upon an agar surface where their numbers and configuration would not be influenced during drying. They were then fixed with osmium tetroxide and pseudoreplicas prepared by the method used previously(4,11).

Results. The findings are illustrated in the electron micrographs of Fig. 1-3. As already described(2), concentrates approaching homogeneity with respect to the kind of particles suspected of being the virus are difficult to obtain. Although the predominant component may clearly represent a population of particles of fairly uniform appearance, there is always present some or much amorphous material of indeterminate characters as well as many particles smaller than those characteristic of erythroblastosis. These properties are exhibited by the concentrate of Fig. 1 which represents one of the best thus far obtained.

Treatment of such a concentrate with the immune serum as described results regularly in agglomeration of particles as seen in Fig. 2. The concentrate was distinctly opalescent before addition of the immune serum. Visible precipitation, that is, the formation of flocs and settling, did not occur when the serum was added, but the suspension was definitely





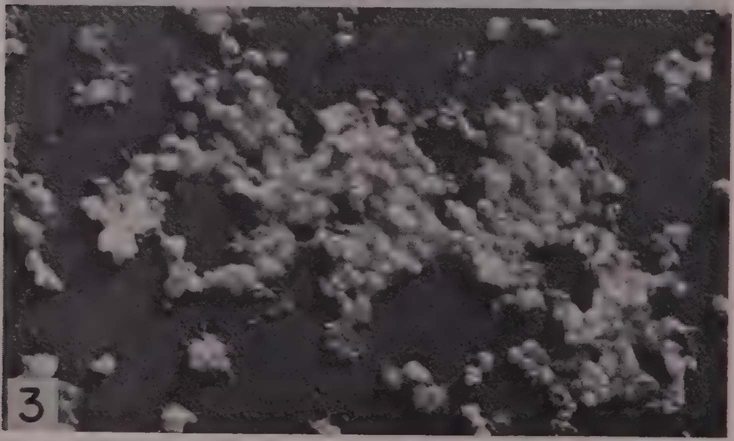


FIG. 1. Electron micrograph of concentrate of particles present in the blood plasma of chickens with avian erythroblastosis (× 15,100).

FIG. 2. Concentrate treated with immune serum for 2 hr at 37°C and for 18 hr at 2-5°C. Concentration of NaCl was 7.5% (× 15,100).

FIG. 3. A portion of Fig. 2 at higher magnification (× 25,000).

more turbid than mixtures of the same particle concentration containing serum from normal chickens. The electron micrograph of Fig. 2 reveals unequivocal agglomeration which did not occur in the untreated concentrates or in concentrates treated with normal serum. The nature of the particles included in these small flocs is shown in the picture of greater magnification in Fig. 3. It is evident that the particles involved are those of relatively uniform appearance described earlier (*2*) as characteristic of avian erythroblastosis. There remain in suspension particles of the same appearance which did not form flocs with the immune serum.

Discussion. Repeated experiments have shown, as will be described later, that the chicken immune serum employed in the present work contains antiviral immune bodies which neutralize the agent of erythroblastosis. Antibodies to this agent, which were produced by relatively small amounts of antigen inactivated with formalin, were less effective than were analogous antibodies to myeloblastosis against the homologous virus. This constitutes, probably, the principal explanation of the partial precipitation in erythroblastosis as compared with the results of similar experiments in which the virus of myeloblastosis could be precipitated quantitatively(4) with its specific avian antibodies. Incomplete precipitation is observed, also, with myeloblastosis with the use of dilute immune serum, the results being essentially identical with those found here in erythroblastosis in which there is excellent reason to suspect that the immune serum was much "weaker" than that obtained with myeloblastosis. It is possible that some of the particles had lost the capacity to react with the immune serum because of antigenic degradation either in the blood plasma or during preparation of the concentrates. This seems rather remote, however, since the agent has appeared fully as stable as the virus of myeloblastosis. In Fig. 2, the presence of individual particles in the smaller size range is notable. Possible relations of the smaller particles to the specific agent are obscure. Many are present in the plasmas of birds with erythroblastosis. Correlative sedimentation studies and particle counts, however, have shown that infectivity is associated principally with particles of approximately 120 mμ diameter. With respect to the present experiments it would appear that, insofar as the precipitin reaction with homologous antiviral immune serum from the homologous host is an acceptable criterion, it can be concluded that the particles characteristic of the disease, as described before and seen in the present pictures, are the virus of avian erythroblastosis.

The results of the present experiments are of significance in several respects. (*1*) They have demonstrated that in avian erythroblastosis specific precipitating (and neutralizing) antibodies can be produced in the homologous host with sufficient quantities of formolized antigen. (*2*) Electron micrographic study of the precipitin reaction provides a specific serological technic for the identification of particles existing in concentrations and amounts too small for adequate study by the usual physical and chemical criteria. (*3*) It is evident that the virus particles of erythroblastosis are indistinguishable by electron micrography from those of the virus of myeloblastosis. This is of critical importance since it has been shown in experiments to be reported later that the agents are distinct chemical and biological entities.

Summary. Spheroidal particles of about 100-120 mμ diameter occur characteristically in the plasma of chickens with erythroblastosis. With formalin-inactivated concentrates of these particles, immune serums have been produced in the chicken which effectively neutralize the infectivity of the agent. These antiserums also cause agglomeration of particles in concentrates identifiable by electron micrography as those found characteristically in this disease. The results have been interpreted to indicate that the characteristic particles agglomerated constitute the viral agent of avian erythroblastosis.

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Comparison of Effect of 'Valmid'* and Other Hypnotics on Brain Respiration *in vitro*. (22611)

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It has been reported that hypnotics in relatively low concentrations are capable of inhibiting the respiration of cerebral slices during electrical stimulation(1), stimulation due to an excess of potassium or to the addition of 2, 4-dinitrophenol(1,2), and stimulation by the removal of calcium ions(3). Ghosh and Quastel(2) interpreted their data as indicating that the inhibition of brain slice respiration by narcotics is restricted to that aspect of cortical respiration which is stimulated by potassium ions.

This study was undertaken to compare the ability of 'Valmid', a new nonbarbituric hypnotic(4,5,6), to inhibit the potassium-stimulated respiration of slices of brain cortex with that of 'Seconal Sodium'† and that of another nonbarbiturate, urethane. Included in this study was a convulsant barbiturate (sodium ethyl, 1, 3-dimethylbutyl barbiturate), (7) serial No. 09757, which has been shown to be capable of uncoupling oxidation from phosphorylation(9).

Method. All experiments were carried out with the Warburg manometric apparatus. Female albino rats of 150-200 g were sacrificed by decapitation, the brains were quickly removed, and freehand slices were prepared by the method of Deutsch(10). Slices totaling approximately 12 mg dry weight were placed into 2.4 ml Krebs-Ringer phosphate medium containing 0.02 M glucose in the main compartments of the vessel. The hypnotic agent, dissolved in 0.3 ml of 25% polyethylene glycol 200 (PEG 200) made up in Krebs-Ringer phosphate, was added to the vessel contents to give final concentrations equivalent to the anesthetic dose (AD_{50}) or 10 times the AD_{50} of each compound, and a final concentration of 2.5% PEG. The concentration of No. 09757 was based upon its convulsive dose (CD_{50}). A control flask was prepared to contain 2.5% PEG 200. PEG 200 was necessary to keep 'Valmid' in solution throughout the experimental period. The sidearm of each vessel contained 0.3 ml M KCl in Krebs-Ringer phosphate. The center wells contained 0.2 ml 10% KOH. Oxygen was used for the gas phase.

After gassing and thermal equilibration, oxygen uptake was measured for one hour to establish control values. At the end of this period the sidearm contents were tipped into the main compartments and oxygen uptake was measured for another hour. Control experiments showed no difference between first and second hour Q_{O_2} values. Readings were taken at 15 minute intervals throughout the experiment.

Results. All values in Table I represent the mean values of 8 experiments. Inhibitions were calculated as percentage inhibi-

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* 'Valmid' (Ethinamate, Lilly) is ethinylcyclohexyl carbamic acid.

† 'Seconal Sodium' (Secobarbital Sodium, Lilly) is sodium allyl, 1-methylbutyl barbiturate.

TABLE I. Effects of Hypnotic Agents on Normal and KCl Stimulated Respiration of Rat Brain Cortex. All flasks contained 2.5% PEG 200 unless otherwise noted. The low concentration for each compound is equivalent to anesthetic dose of compound (convulsive dose in case of No. 09757).

		Without added KCl		With added KCl (0.1 M)		
Hypnotic	Concentration	Q_{O_2}	% inhibition	Q_{O_2}	% inhibition	% stimulation by added KCl (0.1 M)
Control	Without 2.5% PEG	12.38	—	21.76	—	76
	With 2.5% PEG	8.75	—	13.19	—	51
'Valmid'	6×10^{-4} M	7.85	10	10.00	24	27
	6×10^{-3} M	.67	92	.93	93	39
'Seconal sodium'	1.5×10^{-4} M	6.28	28	7.15	46	14
	1.5×10^{-3} M	.96	89	1.91	85	99
Urethane	1.4×10^{-2} M	8.94	0	13.58	0	52
	1.4×10^{-1} M	6.67	24	8.77	33	31
No. 09757	5.7×10^{-5} M	8.88	0	12.27	7	38
	5.7×10^{-4} M	1.35	86	1.12	91	0

tions of the control containing 2.5% PEG 200. 'Seconal Sodium', in a concentration equivalent to its AD_{50}, caused a 28% inhibition of the oxygen uptake of "resting" tissue. None of the other compounds appreciably suppressed respiration when employed in this concentration. In the presence of added KCl 'Seconal Sodium' inhibited respiration by 46% and 'Valmid' by 24% when they were employed in concentrations equivalent to their respective AD_{50}'s. Neither urethane at its anesthetic dose nor No. 09757 at its convulsive dose caused a depression of oxygen uptake of either the "resting" tissue or the KCl-stimulated tissue. In concentrations equivalent to 10 times their respective AD_{50}'s (CD_{50} in the case of No. 09757) all compounds suppressed the respiration of both normal and stimulated slices, approaching 100% in the cases of 'Seconal Sodium', 'Valmid', and No. 09757. However there was practically no increase in the amount of inhibition in the potassium-stimulated slices over that in the normal slices by this higher dosage.

The last column of Table I shows the ability of the hypnotic agents to interfere with the ability of the brain slices to respond to stimulation by an excess of potassium ion. In the presence of only PEG 200 there was a 51% increase in oxygen uptake in response to the increase in potassium. When an anesthetic concentration of 'Valmid' or 'Seconal Sodium' or a convulsive concentration of No. 09757 was also added there was a suppression of the response to KCl stimulation. Urethane induced a corresponding inhibition only when present in a concentration equivalent to 10 times its anesthetic dose. At their respective higher concentrations the other agents so completely depressed the normal "resting" respiration that no significance could be attached to the slight changes caused by the potassium addition.

Discussion. The degree of depression of brain slice respiration produced by a drug employed in a concentration equivalent to its anesthetic dose has been proposed as an index of the narcotizing potency of the drug(11). If this concept is applied to the above data, 'Valmid' appears to possess approximately one-half the potency of 'Seconal Sodium', and No. 09757 and urethane exhibit still lesser potencies. The hypnotic-like action of the convulsant barbiturate in this system is not surprising in view of its chemical structure and in view of the findings by Domino *et al.* (8) that it has depressing as well as stimulating actions. Furthermore, Swanson and Chen (7) suggested that the convulsant effect was probably of spinal origin since its occurrence could be prevented by pithing whereas decerebration and medullary destruction were without effect. 'Valmid' at physiological concentrations in this system exhibited an effect which appears characteristic of narcotizing agents, *i.e.*, a slight inhibition of the respiration of normal slices and a significant depression of the oxygen uptake of potassium stimulated slices. This apparent interference of narcotics with respiration is not taken to indicate that the mechanism of their depressant action is an interference with oxidative processes. On the contrary, the decreased oxygen uptake may be a result of an inability of anesthetized brain cortex slices to utilize the products of cellular oxidations, especially in view of the fact that high energy phosphate has been shown to increase during the anesthetic state(12). The depression of the response to potassium stimulation then would be due to a decreased ability of narcotic-depressed tissue to respond to such stimulation rather than to a narcotic suppression of the subsequent increased respiration. In this respect it is noteworthy that Grenell *et al.* (13) were unable to find convincing evidence that the net synthesis of adenosinetriphosphate was a crucial factor in narcosis or that narcotic action was the result of a decreased oxygen uptake.

Summary. 'Valmid', a new nonbarbituric hypnotic, has been compared with 'Seconal Sodium', urethane, and a convulsant barbiturate with respect to its ability to inhibit normal and potassium-stimulated respiration of rat brain cortex slices *in vitro.* In concentrations equivalent to those obtained during anesthesia, only 'Seconal Sodium' depressed the oxygen uptake of normal tissue whereas both 'Seconal Sodium' and 'Valmid' inhibited the respiration of KCl-stimulated tissue.

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A Revised Method for Isolation of *Schistosoma mansoni* Eggs for Biological Experimentation.* (22612)

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Many of the pathologic, immunologic and clinical manifestations of Manson's schistosomiasis appear to be caused by the presence of the egg stage of the parasite in the host tissues(1,2,3,4,5,6,7). Consequently, studies of the egg stage and its pathogens, independently from the cercarial and adult stages, are assuming increasing importance in understanding the disease. We have developed a method of isolating *Schistosoma mansoni* eggs in adequate quantities and in pure enough state to allow for biological assays *in vitro* and *in vivo.* Our method was developed as a modification of comminution and filtration technics(3,8) with the addition of differential sedimentation through media of differing viscosities whereby gross contaminants may be separated and removed.

Materials and methods. Golden hamsters (9) are exposed to approximately 300 cercariae of *Schistosoma mansoni.* Eight weeks after exposure to infection one or 2 hamsters are sacrificed and stored at ordinary refrigerator temperature for 24 or 48 hours. All

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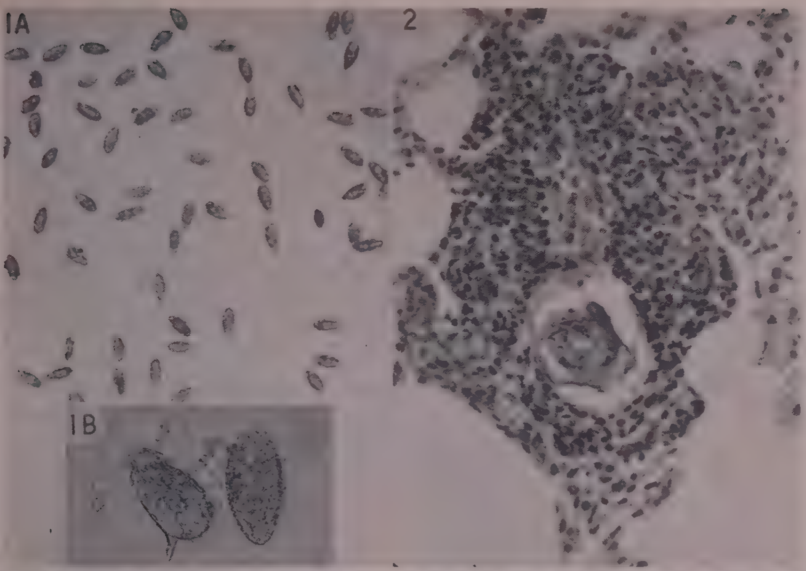

FIG. 1A. Sample of schistosome eggs isolated from hamster liver by method described in text. 40×.

FIG. 1B. Circumoval precipitates around the "purified" egg after 24-hr incubation in human immune serum. 180×.

FIG. 2. Cellular reaction around a schistosome egg in lung of an experimental mouse 30 days after injecting the mouse intravenously with purified eggs. 200×.

subsequent operations can be carried out at room temperature. The livers are removed from the animals and comminuted for 2 min. in 150 ml 1.7% NaCl aqueous solution in a household model Waring blendor. The concentration of saline used is sufficient to prevent premature hatching of the eggs without affecting them adversely(3). The homogenate is poured through 2 nesting wire mesh filters (phosphor bronze screening of 50 and 80 mesh) into a conical glass settling flask of 6 in. depth and 275 ml capacity, flushing the screens with added quantities of the saline sufficient to fill the cone. Thirty minutes later the contents of the flask are carefully decanted until about 1¼ in. remain. This remainder, containing the settled eggs and some contaminating material from the liver, is transferred to a centrifuge tube and washed 4 or 5 times in 1.7% saline with very mild 1½ min. centrifugation between washes. The resulting rough-cleaned sediment is concentrated in about 5 ml of saline, agitated with a pipette to break up adhering clumps, and then very carefully overlaid (using a fine pipette) upon a filtered solution of freshly prepared 0.5 molar sucrose in a standard 50 ml burette, the capillary tip of which has been removed. If observation of falling particles is desired, a dissection microscope is mounted in a horizontal position and focused at the lowest centimeter of the burette column.

Settled particles heavier than the eggs may be drawn off in 3-5 ml, and discarded, at the end of 15 min. At the end of one hour, 15 ml are drawn off into a small centrifuge tube. This tube contains the bulk of the eggs which are then washed free of sucrose. and concentrated, in 1.7% saline, using very light centrifugation. If further purification is desired the concentrate is carefully flushed over a small piece of 325 mesh screen to remove particles smaller than the eggs, and the eggs are then flushed off the top of the screen into a suitable container.

Results. If scrupulously cleaned containers and filtered solutions are used, and if the entry of extraneous dirt and dust is prevented, the purified eggs in the final concentrate are practically free of foreign material (Fig. 1a), occasionally even without the final screening step. They maintain their morphologic integrity (Fig. 1b). When placed in distilled water at the end of the treatment, many of them hatch rapidly producing actively swimming miracidia. They retain their acid-fast property(10) and the normal antigenic quality of reacting with circumoval precipitins in human immune serum(3, Fig. 1b). After bringing the eggs into physiological salt solution we have injected them intravenously(11, 12) into mice, finding that the mice survived for at least 30 days and that there was a host cellular response to the injected eggs trapped in the lungs(11, Fig. 2). Eggs recovered from the upper portion of the column of sucrose, after one hour, are few in number, tend to be much smaller, immature, of bizarre structure and reduced hatchability, or else are empty.

Discussion. The technic outlined above offers a fairly rapid method of isolating *Schistosoma mansoni* eggs, in a relatively pure state, from hamster livers. The method can be adapted and refined to suit a variety of research purposes and source materials of eggs. We experimented with infected hamster livers of various degrees of freshness and found that best results were obtained from "stale" livers, that is those in which postmortem autolysis had been given an opportunity to occur through one or 2 days of storage of the killed animals at refrigerator temperature. When fresh livers were used, there was often an increased amount of tissue debris in the original homogenate and a tendency for the eggs to agglutinate or become enmeshed with tissue particles so that separation was incomplete. It may be, however, that the use of stale livers, or the passage of the eggs through glucose, may alter the eggs in their suitability for tests other than those which we used. Livers with appreciable amounts of fat were generally unsuitable.

Summary. A simple procedure has been outlined for isolating Schistosoma eggs from host liver tissue by a combination of comminution, filtration, and sedimentation through a viscous medium (sucrose). The resultant purified eggs retain certain normal qualities tested such as morphological integrity, hatchability, acid-fastness, ability to react with circumoval precipitins in immune serum, and ability to incite tissue reactions when injected into experimental animals. The procedure also provides for a degree of separation of fully mature eggs from other types, and for microscopic observation and some degree of control of the separation process.

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In vivo Release of Radiocarbon from Labeled Ehrlich's Ascites Carcinoma Cells. (22613)

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The relationship between tumors and their host tissues, particularly with respect to protein metabolism, has been the subject of numerous investigations(1-4). Recently, isotopic experiments have been reported in which the administered isotope was confined to the transplanted tumors themselves(5,6). These studies have suggested that protein metabolism in tumors is essentially, though not strictly, a one-way passage. Babson and Winnick(5) transplanted Walker carcinoma tissue labeled with tyrosine or leucine-C^{14} into rats. A rapid initial loss of radioactivity was observed during the first day, resulting from cell breakdown during fragmentation and from initial autolysis. A small but significant amount of the remaining radioactivity was released in each of the following 5 days. Greenlees and LePage(6) injected TA 3 ascites cells, labeled *in vivo* with C^{14}-glycine, into the peritoneal cavity of normal mice. Radioactivity was released from the tumors with 9% appearing daily in various organs, urine and respiratory CO_2. The transfer of radioactivity from the tumor to the host tissues suggested that the loss of isotope from the tumor cells was continuous and regular. Since the tumor proteins were not separated and analyzed in these experiments (6) and radioglycine is known to contribute C^{14} to the lipides and nucleic acids, an exchange between the neoplasm and its host strictly with regard to amino acids cannot easily be interpreted.

In the present study, the release of radioactive protein from Ehrlich's mouse ascites carcinoma cells labeled with C^{14}-leucine was investigated. Radiocarbon from leucine is incorporated primarily into the proteins; however, since some of the radioactive products from leucine were taken up by the lipides and nucleic acids, analyses of these components are also presented.

Experimental. Ascites cells labeled with radiocarbon were prepared *in vivo* by intraperitoneal injections of leucine-2-C^{14} into mice bearing a 4-day-old ascites tumor. One mg of radioleucine with a specific activity of approximately 4.5 x 10^6 counts per minute (C.P.M.) per mg was injected per mouse. The radioactive cells were recovered 48 hours after injection of the isotope, separated from the suspending ascitic fluid by centrifugation, and resuspended in 10 volumes of Krebs-Ringer-Phosphate buffer solution, pH 7.4. Aliquots of 1 ml of the cell suspension were injected into the peritoneal cavity of normal mice. At the end of the experimental period the cells were recovered with the use of several 0.9% saline washes and separated by centrifugation.

The acid-soluble (TAS), lipide, nucleic acid and protein fractions were obtained by the procedure of Hurlburt and Potter(7) as modified by Tyner *et al.*(8). Duplicate aliquots from all samples were plated directly and counted with a gas-flow tube. Proteins were plated from a suspension in ethanol-water. A minimum of 4,000 counts were taken and the appropriate self-absorption corrections applied.

Results. The recovery of labeled transplanted tumors removed from the animal about 15 minutes after inoculation is presented in Table I. Approximately 20% of the administered radioactivity could not be recovered immediately after inoculation. Such losses, probably due to manipulation, experimental technic, etc., were consistent but greater than the 8% observed by Greenlees and LePage(6). In all of the following experiments, one animal was sacrificed immediately after inoculation and recoveries at subsequent times are expressed as per cent of the radioactivity recovered after 15 minutes.

Table II summarizes the results obtained with radioactive ascites cells maintained intraperitoneally for 24 and 48 hours. Consid-

TABLE I. Recovery of Radioactivity from Labeled Ascites Cell Transplants Withdrawn Immediately after Inoculation.*

	Exp. A			Exp. B		
Fraction	Original	Withdrawn in 15 min.	% recovered	Original	Withdrawn in 15 min.	% recovered
TAS	4,180	3,175	76	1,080	745	69
Lipides	940	1,020	—	216	200	93
Nucleic acids	2,140	1,750	82	459	354	77
Proteins	90,200	72,000	80	13,400	10,600	79

* Total C.P.M. per fraction.

erable tumor growth occurred as shown by the increase in the weight of tumor protein recovered. The lipides did not change consistently or significantly in any of the experiments. Twenty-four and 45% of the radioactivity in the nucleic acid and protein fractions respectively was lost from the tumor during the first day. Less than 10% of the remaining radioactivity in either of these 2 fractions was lost during the 2nd day. The release of isotope from the ascites cells is considerably slower and constant between the 2nd and the 5th day (Fig. 1). After a rapid initial loss during the first day, an average decrease of about 8% per day was observed in the nucleic acids and proteins recovered. The possibility that the marked loss of radioactivity during the first day may have been due to a reaction of the host against the inoculated tumor, resulting in a destruction of tumor cells, was tested by transplanting the labeled tumor into a mouse already bearing a tumor. Three days after inoculation of a mouse with 0.2 ml of an unlabeled tumor cell suspension, the isotopic ascites cells were injected. An aliquot of the labeled preparation was injected into a non-tumor-bearing mouse at the same time. The results are shown in Table III. No significant differences were found in the release of radioactivity from the 2 inocula. Over 30% of the C^{14} in the nucleic acids and proteins of both tumors was lost during the first 24 hours. This rapid initial release may reflect the metabolic behavior of the transplanted cells. The possibility exists that a number of the cells in the preparation may be incapable of survival and growth, may autolyse, and are thus lost from the transplant soon after inoculation. Babson and Winnick (5) found that between the 2nd and the 5th day, the actively growing isotopic tumor transplants in their experiments released approximately 8% per day of the initial radioactivity administered. The authors cite as a possible criticism of their experiments that

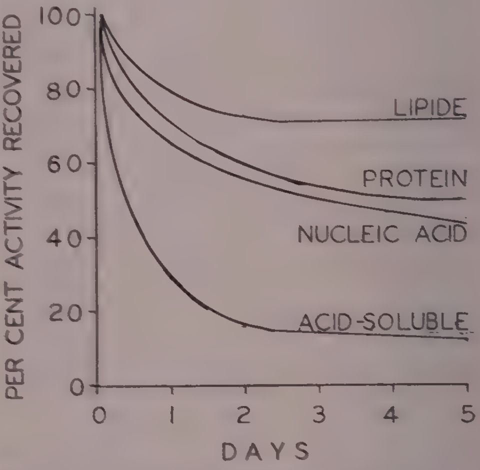

FIG. 1. Recovery of radioactivity in various fractions of labeled tumor transplants maintained intraperitoneally for 5 days. Values are expressed as % of activity recovered 15 min. after inoculation.

TABLE II. Recovery of Radioactivity from Labeled Transplants Maintained for 2 Days.

	TAS		Lipides	Nucleic acids		Proteins		
Time	C.P.M.	% recovered	C.P.M.	C.P.M.	% recovered	C.P.M.	% recovered	Wt of protein (mg)
15 min.	3,175		1,020	1,750		72,000		23.9
24 hr	2,490	79	1,250	1,320	76	40,000	55	79.6
48 "	1,860	69	1,340	1,205	69	37,200	52	90.2

TABLE III. Influence of Pre-Existing Tumor on Release of Radioactivity from Labeled Transplants.*

	Normal mice			Tumor-bearing mice		
Time, hr	TAS	Nucleic acids	Proteins	TAS	Nucleic acids	Proteins
24	53	68	60	42	71	68
48	45	61	55	39	60	64

* Recoveries of radioactivity expressed as % of 15 min. values.

since the most rapid growth occurs at the periphery of the tumor, the original transplanted portion would probably be the first to become necrotic. Very little necrosis was observed however. Greenlees and LePage (6) found that labeled TA3 ascites cells released 10 to 20% daily of the radioactivity originally present. These workers present analyses of the combined lipide, nucleic acid and protein fractions from the recovered cells which showed a loss of 11% of the C^{14} on the first day and 60% in 2 days. The extent to which C^{14} is released from any of these components and thus contribute to the body pool cannot be estimated from this data. The present report indicates that actively growing Ehrlich's ascites cell proteins release about 8% of their radioactivity per day during the 2nd to the 5th day period.

The data presented here and that quoted above suggest that a small but significant exchange exists between the proteins of the tumor and its host. The possibility exists that the failure to recover all of the administered isotope may be due not to the metabolic release of labeled constituents from the ascites cells but to the loss of cells due to autolysis.

Summary. Ascites cells labeled with radiocarbon from leucine-2-C^{14} were injected into mice. The amounts of radioactivity remaining in the acid-soluble, lipides, proteins and nucleic acids of the transplants for periods up to 5 days was measured. A marked loss of C^{14} from all of these components occurred within the first day but did not appear to be due to a reaction of the host against the inoculated cells. Between the 2nd and the 5th day a small but significant amount of the C^{14} in the proteins and nucleic acids was lost per day. These results suggest that the transfer of labeled nitrogenous constituents from the tumor to the host tissues occurs to some extent. The possibility that these results may be due to the destruction of the cells must also be considered.

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Effect of Levan on Transmitted Mouse Leukemia and on Ehrlich Ascites Tumor Implants.* (22614)

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Evidence is accumulating that leukemia in mice may be caused by a filtrable, submicroscopic agent, presumably a virus(1). Pillemer *et al.* reported that the complement-properdin system neutralizes and inactivates some viruses(2), and that native levan and other high molecular weight polysaccharides alter properdin levels *in vivo*(3). Hestrin *et al.*(4,5) suggested that the infection-promoting activity (IPA) of native levan, injected intravenously or intraperitoneally into mice having an intraperitoneal infection, may result largely from an extraperitoneal interaction between levan and host. Davies and co-workers(6) suggested that this IPA is also related to an endothelium sealing activity (ESA) on the part of the large polysaccharide molecules, which, circulating in the blood, enter into the interstices of capillary endothelium and alter its sieving characteristics toward colloids in an area of inflammation.

Because of (a) the reported anti-viral activity of the complement-properdin system, (b) the IPA of native levan, and (c) the presumed viral etiology of mouse leukemia, the effects of a properdin-lowering substance, levan, on the course of transmitted leukemia was studied.

Based on one preliminary experiment(7) there is some indication that the lowering of the properdin level by high molecular weight polysaccharides may facilitate transplantability of the Ehrlich Ascites Tumor. In view of this and of the phenomenon of ESA, it was desirable to know if levan injected intravenously would either interfere with the passage of nutrients to a rapidly growing tumor, thus inhibiting the establishment of the tumor, or as Koprowski(7) has indicated, facilitate the establishment of the tumor. In addition, the direct action of levan on the Ehrlich Ascites Tumor cell was studied by observing the effect of injecting a mixture of levan and tumor cells subcutaneously or intraperitoneally.

Methods. (a) Leukemia: Male C58 mice, age 2 months, were used. Eleven mice (Group A) were injected intraperitoneally with 5 mg of levan† dissolved in 0.15 M NaCl solution. Group A and 11 control mice (Group B) then received intraperitoneally 0.3 ml of a suspension of lymph nodal cells 25,000 to 30,000/mm^3) from a leukemic C58 mouse. The cells were obtained from a mesenteric lymph node by teasing with forceps and probing needles. The cells were then suspended in a balanced physiologic buffer solution and the suspension was finally filtered through an electron microscope grid mesh. The mice of Group A received intraperitoneally 5 mg of the levan solution every fourth day until palpable peripheral lymph nodes were observed. Including the initial injection, 3 injections of levan were given.

(b) Ehrlich Ascites Tumor: Female or male CFW mice, generally 6 to 8 weeks old, were used,

I. Experimental mice received by tail vein 5 mg of the levan solution. Two to 5 hours later these mice and the controls received subcutaneously 0.3 ml of a suspension of Ehrlich Ascites Tumor cells. The tumor cells were obtained from the peritoneal cavity of mice 7 to 9 days after implantation. Thereafter, the experimental animals received one injection of levan 4 days later, one group by tail vein, another group, intraperitoneally. In the final experiment of this series one group of 11 mice received one levan injection by tail vein 4 days later and 10 mice of another group received the levan by tail vein daily for 1 to 4 days.

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† Levan ("Hestrin IV") was kindly donated by Dr. Louis Pillemer.

TABLE I. Effect of Levan on Establishment of Solid Phase Tumors in CFW Mice Receiving Ehrlich Ascites Tumor Cells Subcutaneously.*

		Levan on 4th day‡		
Exp.	Controls†	I.P. route	I.V. route	
1	4/4	6/6	Not done	
2	6/6	6/6	5/5	
3	7/7	6/6	5/5	
		Levan by I.V. route		
		On 4th day	On days 1 and 4	On days 1, 2, 3, 4
4	12/12	11/11	3/3	7/7

* All mice initially given levan by tail vein, followed by subcut. inj. of tumor cells.

† Numerators = No. of tumors. Denominators = No. of animals in each group.

‡ No. of days after initial injection.

II. An aliquot of a suspension of the tumor cells was mixed with an equal volume of the levan solution and immediately injected into mice either subcutaneously or intraperitoneally. A second aliquot of the tumor cell suspension was mixed with the levan and, after 5 minutes, was similarly injected. Control mice received only the tumor cells, either subcutaneously or intraperitoneally. There were 5 mice in each of these 6 groups.

Results. (a) Leukemia: Palpable peripheral lymph nodes began to appear in both control and levan-treated mice 7 days after the injection of the suspension of leukemic cells. Two days later one levan-treated mouse was dead and all the remaining 21 mice had palpable peripheral lymph nodes. The levan-treated mice all died 9 to 13 days after the injection of cells, while the controls died 12 to 16 days after the injection of cells.

(b) Ehrlich Ascites Tumor: I. From Table I it can be seen that the injection of levan did not inhibit the formation of tumors: All mice, control and levan-treated, developed tumors at the site of inoculation of the Ehrlich Ascites Tumor cells. All of these animals were kept 2 weeks or longer. No tumors were observed to regress. In addition, there was no apparent enhancement of tumor formation.

II. The direct mixing of levan and the tumor cells and their subsequent injection, immediate or delayed, did not prevent tumor growth in any of the mice injected either by the subcutaneous or intraperitoneal routes. All subcutaneously injected mice developed tumors at the site of inoculation. One of the levan-treated mice died 7 days after the injection. It had a small tumor at the site of inoculation. The remaining 14 mice were sacrificed 54 days after the injection and no tumor was observed to have regressed. Of the 15 mice receiving the intraperitoneal injection, 14 developed ascites and died from 9 to 26 days after the injection, which is not uncommon among CFW mice used for serial transfer of this tumor. One mouse had been killed 7 days after the injection and its tumor cells served as seed for serial transfer. In Table II are the survival times of the mice of the 3 groups.

Discussion. (a) Leukemia: In this laboratory the transmission of leukemia in C58 mice has always been 100% successful. Since mice injected with the same suspension of leukemic cells may succumb to leukemia over a period of 7 days or more, the slight difference in the times of death of the controls and levan-treated animals is not considered significant. From this experiment it is apparent that the course of the leukemia was not altered by the levan.

(b) Ehrlich Ascites Tumor: This tumor had been implanted by intraperitoneal injection 59 times in CFW mice prior to the beginning of these studies. It was introduced in this laboratory by one of the authors (W.B.W.) after it had been maintained for several years in the Stocker strain of Swiss Albino mice. After subcutaneous injection into CFW mice the ascites cells give rise to solid phase tumors at the site of inoculation. During the course of these experiments 126 mice, including the 78 listed in Table I, were injected subcutaneously with Ehrlich Ascites Tumor cells. One hundred and fourteen (90%) mice developed tumors, while 12 mice failed to develop tumors. None of these tumors was observed to regress. Since tumor establishment was not inhibited by the levan, whether injected independently of or after direct contact with the tumor cells, it would seem that the postulated ESA of the levan had no effect on tumor development in this study. The effect of repeated levan injections on the properdin level is unknown. From Table I it is seen that the results were

TABLE II. Time of Death, after Intraperitoneal Injection, of Mice Bearing the Ehrlich Ascites Tumor.

Mouse	Days after inj.
Control	7*
"	9
A	15
Control	16
A	16
A	16
B	16
B	16
B	18
A	18
B	20
B	20
Control	20
A	21
Control	26

* Killed. Used as seed for serial transfer.
A = Levan-cell mixture inj. immediately.
B = " " " " after 5 min.

the same with 2 to 5 injections (including the initial injection) of levan. The incidence of successful intraperitoneal transmission of the Ehrlich Ascites Tumor in CFW mice is greater than 98% in this laboratory: Of 271 mice used for its serial transfer not more than 3 unsuccessful transfers have been encountered and none since the forty-sixth transfer. Because of this high incidence of successful transfers, this form of the tumor was considered satisfactory for study of the effect of levan on tumor cells after direct mixing. Since survival times of the mice in the 2 experimental groups listed in Table II were not prolonged as compared to the controls, it would appear that the levan did not prevent the establishment of the ascites form of the tumor.

Summary. Injections of levan intraperitoneally into C58 mice did not alter the course of transmitted leukemia. Levan injected by tail vein or intraperitoneally failed to inhibit and did not apparently enhance establishment of a solid phase tumor at the site of inoculation of Ehrlich Ascites Tumor cells. The direct action of levan on the tumor cells injected immediately or after a 5 minute delay did not result in inhibition of either solid phase tumors or the ascites tumor.

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Platelets XVIII. Relationship of Platelets to Activity of 5-hydroxytryptamine Creatinine Sulfate.* (22615)

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It was originally thought that 5-hydroxytryptamine creatinine sulfate (5-HT) is an intimate constituent of platelets(1,2). This concept has more recently been revised to show that, like other factors(3), 5-HT may be only absorbed to platelets(4). However, the exact role of platelets in the metabolism of 5-HT remains controversial.

We have recently shown that 5-HT has a powerful local venopressor effect. Thus, measurement of local venous pressure is a simple and reliable method, detecting as little as 0.3 γ/5-HT administered in 1 minute time (5). In an effort to elucidate the relationship between 5-HT and platelets this technic was then applied to the study of the effects of incubation of 5-HT with various platelets preparations on the activity of the substance.

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Materials and methods. Individuals with normal peripheral venous pressure were subject of this study. Changes in venous pressure were evaluated by means of the direct H_2O manometric method previously described (5). Crystalline 5-HT[§] was diluted in saline solution prior to its use or its incubation with platelets, platelet products, plasma or serum. Human plasma was separated by centrifugation from whole blood collected in plastic bags containing standard ACD solution;[‖] platelet-rich plasma was obtained by centrifuging whole blood at 500 rpm/30′/4°C; platelet-poor plasma by centrifuging at 3,000 rpm/30′/4°C. Serum was separated by centrifugation from whole clotted blood incubated for 6 hours at 37°C. Platelet suspensions in saline solution were prepared as previously described (6). Platelets, however, were washed only once to limit their damage and greatly concentrated so that platelets from 500 ml blood were resuspended in 15 ml of saline only. All fractions were obtained and used within 8 hours after collection of blood. Silicone-coated glassware, Arquad 2-C coated needles and plastic tubing were used in all experiments with platelets. Other technics used are described when appropriate.

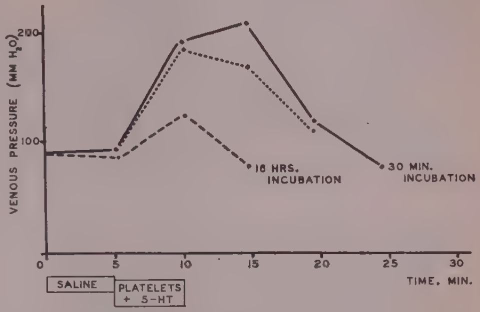

FIG. 1. Effect of short and prolonged incubation with intact platelets on venopressor activity of 5-HT.*

* A typical experiment. Platelet suspension was incubated with 5-HT for 30 min. or 16 hr at 4°C (see text). Dotted line shows response to the same dose of 5-HT by the same subject. Slight enhancement of venopressor effect of 5-HT through short incubation with platelets remains unexplained (see text).

Results. Preliminary observations showed that time of incubation was most important in determining the effect of various agents on the activity of 5-HT. Short and long incubation experiments were then run in each study. All experiments were performed in 4 different subjects. (a) *Venopressor activity of 5-HT after* short-time incubation *with fresh serum, platelet-rich plasma, platelet poor plasma and saline suspensions of platelets* (Fig. 1). One tenth gamma of 5-HT per ml was added to each preparation. A solution of 5-HT containing 0.1 γ/ml in physiological saline represented the control. Mixtures and control were incubated at 4°C for 30′. Fifteen ml of each preparation were then injected over a 5 minute period, at a speed of 3 ml/minute, one single subject receiving all preparations to reduce individual variability of results. Saline solution was given at the same speed between injections for periods of 15 minutes. *Fresh*[¶] plasma, serum and suspensions of intact platelets had no venopressor activity. Saline solutions of 5-HT, fresh serum, fresh platelet-rich and platelet-poor plasma containing 5-HT kept full activity. Activity of 5-HT was enhanced after incubation with suspensions of platelets (Fig. 1). (b) *Venopressor activity of 5-HT after* prolonged incubation *with fresh serum, platelet-rich plasma and platelet-poor plasma* (Fig. 1). Mixtures of 5-HT and these various preparations were incubated at 4°C for 16 hours. None of the preparations tested showed venopressor activity. A control solution of 5-HT kept at 4°C for 16 hours had full activity. (c) *Interaction of 5-HT with various platelet preparations.* Once washed platelets from 1 pint of blood were resuspended in 15 ml of saline. Following incuba-

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[§] Kindly supplied by Dr. John Webb, Upjohn Co., Kalamazoo, Mich.

[‖] Fenwal Laboratories, Framingham, Mass.

[¶] Recent observations in this laboratory have indicated that *stored* plasma contains factors able to mimic the local venopressor effect of 5-HT.

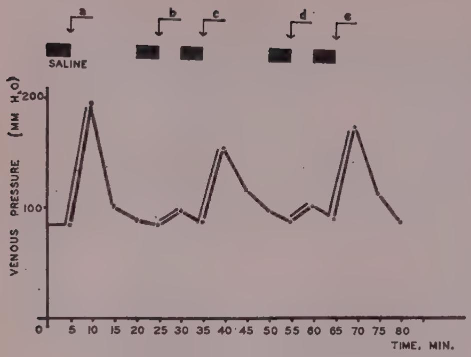

FIG. 2. Venopressor effect of various preparations.*

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* (a) Saline solution of 5-HT (0.1 γ/ml); (b) intact platelets; (c) "platelet protein solution"; (d) supernatant, after incubation of 5-HT and platelets (16 hr); (e) "platelet protein solution" from platelets previously incubated with 5-HT.

Avg values from 3 sets of experiments supplied data for preparation of this composite chart. In actual experiments, longer intervals of time were used, and some experiments were carried out on different days.

tion with 1.5 γ of 5-HT for 16 hours at 4°C mixture was centrifuged at 3,200 rpm/30 min./4°C. Supernatant was then transferred by suction to another container and platelets were resuspended by gentle shaking in 15 ml of fresh saline solution. Both supernatant and platelets failed to show venopressor activity (Fig. 2). To determine whether 5-HT had been entirely inactivated, destroyed, or, rather, incorporated by platelets, the following experiment was carried out. Two buttons of platelets were prepared simultaneously from 2 units of 250 ml of blood from the same donor. One was dissolved in 15 ml of physiological saline; the other in 15 ml of saline containing 0.1 γ/ml 5-HT (total 1.5 γ); both mixtures incubated for 16 hours at 4°C. In each instance, mixture was then centrifuged in the cold at 3,200 rpm/30′, supernatant separated and platelets resuspended without washing in 1 ml of saline. Three ml of ethyl ether (which does not remove or destroy 5-HT) were added and mixture shaken vigorously for 5 minutes to obtain disruption of platelets(7). Mixture was then centrifuged at 3,200 rpm/10 minutes, supernatant ether discarded by suction, leaving behind a "*platelet-protein solution*". An additional reagent in this series of studies was saline solution containing 0.1 γ/ml. 5-HT, kept at 4°C for 16 hours.

All preparations were separately injected in healthy individuals with following results (Fig. 2): (a) 5-HT solution gave the expected venopressor response; (b) protein solution from platelets and platelets incubated with 5-HT gave a slightly less pronounced but still significant venopressor response.

Discussion. Fate and function of 5-HT are closely related to platelets, but the details of this relationship remain somewhat obscure. Since the mature platelet cannot form 5-HT from its precursor 5-Hydroxytryptophane (8), the substance may not be produced in platelets. Most commonly accepted theory is that 5-HT is produced in the enterochromaffine cells, then released into the blood stream. Absorbed by platelets, it is carried to various districts(9). Absorption by thrombocytes may represent a system of convection, as well as a mechanism of protection for 5-HT, since aminoxidases are present in plasma. If *in vitro* experiments with synthetic 5-HT duplicate the behavior of natural substance *in vivo,* our studies elucidate certain aspects of the relationship between 5-HT and platelets. Short time incubation with fresh serum, plasma and platelets did not inactivate 5-HT; long term incubation did. In case of plasma and serum, degradation of 5-HT may have occurred on long incubation; in the case of platelets an alternative explanation offered itself, absorption or binding of 5-HT by thrombocytes. This hypothesis received support from some of our findings, since, following prolonged incubation with 5-HT, intact platelets did not exhibit venopressor effect; products of disrupted platelets did. Therefore, whatever the nature of absorption or binding between platelets constituents and 5-HT, this was a strong one requiring destruction of the integrity of the platelet body. A complicating feature of these studies was the observation that "platelet protein solution" a product of platelet disruption showed significant venopressor effect, without addition of or incubation with 5-HT. This suggested that platelets contain an appreciable amount of natural 5-HT or, perhaps, of other venopressor agents. Protein solutions from platelets incubated with 5-HT, however, proved to possess higher venopressor effect than from platelets alone, all experimental conditions being equal. Presumably, some 5-HT had been absorbed or bound by platelets.

Summary. 1. 5-hydroxytryptamine creatinine sulfate (5-HT) administered intravenously causes significant elevation of the local venous pressure at doses as small as 0.3 γ/min. This effect is not affected by short-time incubation with fresh serum, platelet-rich and platelet-poor plasma. Incubation for 16 hours causes destruction of 5-HT activity. 2. Activity of 5-HT also disappears after incubation for 16 hours with suspensions of washed platelets. Some of the activity, however, can be recovered after total disruption of thrombocytes. Also a protein solution from human platelets exhibits significant venopressor activity, while intact platelets are inactive. 3. These findings suggest: (a) presence of natural 5-HT or of substances with venopressor effect in human platelets; (b) absorption of 5-HT by platelets upon incubation; (c) strong bondage between 5-HT and platelet constituents.

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Action of Sodium Salicylate on Tissue Gas Tensions.* (22616)

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It is known that after intravenous administration of sodium salicylate (100 mg/kg body weight) ventilation increases secondary to both direct respiratory center stimulation and elevation in metabolic production of CO_2; alveolar CO_2 tension decreases 10%; oxygen consumption increases approximately 70%; arterio-venous oxygen content difference increases by 50%; and cardiac output is elevated by 20%(1).

With the above actions in mind, the question arises as to how tissue gas tensions are altered. Tissue carbon dioxide may be expected to follow alveolar air, but the oxygen tension in tissues is determined by an exacting relationship between the tissue metabolic demand and its blood supply. The problem of measuring gas tensions in tissue was approached through construction and analysis of subcutaneous depots of gas, a technic developed by Campbell(2) and shown to provide an adequate estimate of extracellular, if not intracellular fluid gas tensions.

Methods. Gas depots were formed in the interscapular region of non-fasting albino rats by subcutaneous injection of 20 cc of sulphur hexafluoride, an inert gas of high molecular weight and low blood solubility. Its prolonged disappearance time facilitated sampling and in no way interfered with the equilibrium tensions of CO_2 and O_2(3). Twenty-four hours after depot construction, 2 groups

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TABLE I. Tissue Tensions Albino Rats.*

Category	No.	CO_2 tension, mm Hg	O_2 tension, mm Hg	Stand. error of difference between means	
Control	12	45.4 ± .7 (41.3–49.2)	49.2 ± .8 (44.0–54.0)	CO_2 tension	O_2 tension
Sodium salicylate, 200 mg/kg/24 hr	11	34.6 ± .6 (37.9–49.5)	43.8 ± 1.2 (37.9–49.5)	.9	1.5
" " , 300 "	10	38.2 ± .6 (34.8–40.5)	49.3 ± 1.6 (41.2–57.1)	.9	—

* Mean values ± 1 stand. dev., (range in parentheses).

of rats received sodium salicylate intraperitoneally in divided doses over the next 18 hours. The third group, controls, received no drug. Gas depots were aspirated in all groups 48 hours subsequent to construction and analysed for CO_2 and O_2 by the Scholander-Roughton technic(4). At the time chosen for analysis, though arbitrary, one could anticipate that an equilibrium had been achieved between gas tensions in the subcutaneous depot and tissue fluids, but development of fibrous changes in the tissue had not occurred(2). No evidence of subcutaneous infection was noted in any of the rats employed.

Results. Results are tabulated in Table I. The fall in CO_2 tension finds a ready explanation in the action of salicylate as a primary respiratory stimulant. The resultant decrease in alveolar (arterial) CO_2 tension will be promptly reflected as an almost equivalent decrease in venous blood and tissue CO_2 tension. The absolute decrease in both alveolar and tissue tensions will depend on relative excess of ventilatory drive initiated by salicylate over the action of this drug to increase metabolic production of CO_2. The significant decrease in tissue O_2 tension caused by 200 mg/kg/24 hr of sodium salicylate can be explained on the basis of several mechanisms acting simultaneously. Of primary importance is relationship of regional blood flow to tissue metabolism. By the Fick principle, blood flow is directly proportional to oxygen consumption and inversely proportional to the arterio-venous oxygen content difference. If tissue oxygen tensions were to remain constant in the presence of an increased oxygen consumption initiated by salicylate, increase in blood supply must be equivalent to increase in oxygen consumption. Any failure of the blood supply to meet this demand would necessitate an increase in the arterio-venous oxygen content difference and thus a fall in venous oxygen tension. Since gas in tissues and regional venous blood are in equilibrium(5), a fall in tissue oxygen tension to at least the same degree will result in each. The fall in tissue O_2 tensions produced by salicylate can only mean that there has been a relatively greater increase in O_2 consumption by this drug than in regional blood flow.

The rise in alveolar ventilation responsible for the decrease in alveolar CO_2 tension will result in a simultaneous rise in alveolar oxygen tension. If the alveolar-arterial O_2 tension gradient remains unchanged, this will raise directly the arterial O_2 tension and arterial O_2 content. However, if there were a constant arterio-venous O_2 content difference, the venous oxygen tension would fall slightly because of the effect of CO_2 on the oxygen dissociation curve of hemoglobin (Bohr effect). This mechanism could however, account for only about 3 mm. Hg fall in O_2 tension.

In the dog, the increase in oxygen consumption following large doses of salicylate may be less pronounced than with smaller doses(1) and metabolic depression with high concentrations *in vitro* has been amply confirmed(6,7,8). The difference in tissue oxygen tension response to 200 and 300 mg doses suggests that the metabolic dose-response pattern may be similar in the rat. The CO_2 tension differences between the 200 mg and 300 mg doses similarly implies that a dose-response relationship exists for direct stimulation of the respiratory center.

Summary. In the rat, sodium salicylate in doses of 200 mg/kg/24 hr produced a fall in tissue CO_2 and O_2 tensions, effects due to the increased alveolar ventilation and relatively greater increase of tissue oxygen consumption than tissue perfusion.

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A Study of the Mechanism of the Schultz-Dale Reaction.* (22617)

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(Introduced by Ann G. Kuttner.)

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The Schultz-Dale reaction(1,2), which consists of the contraction of smooth muscle from an antigenically sensitized animal on re-exposure *in vitro* to the antigen, has a manipulative simplicity that makes studies of mechanism particularly inviting. The mechanisms that have been proposed fall into 2 overlapping groups: (1) liberation of a stimulant such as histamine or acetylcholine as a result of the antigen-antibody(3,4,5) and (2) involvement of nerve, a possibility that has been considered both favorably(6) and unfavorably(7,8,9), without very final evidence either way. The literature concerning the effects of pharmacodynamic agents and enzyme inhibitors on this reaction gives useful preliminary information bearing upon questions of mechanism, but the data are scattered and were obtained under a wide variety of experimental conditions. Therefore a preliminary study was made of the effects of a selected group of drugs and enzyme inhibitors upon the reaction, since it seemed likely that indications might be obtained as to which physiological or enzymological systems were involved in the reaction.

Gray, Pedrick and Winne(10), using guinea-pig ileum, found that 0.8% ethyl alcohol blocked the Schultz-Dale reaction, which suggested to us that nerve might be involved, and further experiments to test this possibility were performed. Results that support the hypothesis that nerve is involved are presented below.

Materials and methods. Guinea pigs weighing about 500 g received intraperitoneally 2.0 mg of crystalline egg albumin on 2 successive days. After 21 to 24 days, the animals were killed, and the ileum was removed and washed with Tyrode's solution. Portions of the ileum about 15 mm long were set up in a muscle bath stirred by aeration and arranged for washing by overflow. It was found important to keep the lumen open at both ends. The bath and all solutions were kept at 37.5°C. Contractions were recorded with an ink-writing lever that exerted a tension of about 0.5 g, and gave an amplification of 1:4.5. The usual method of testing the effect of a substance of interest upon the action of antigen and other stimulants was to add the substance to the bath containing the ileum, and after 15 minutes, to add the stim-

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TABLE I. Effect of Various Substances upon the Schultz-Dale Reaction.

Inhibitor	Concentration	Reactivity, in presence of inhibitor, to:		
		Antigen (see text for conc.)	Acetylcholine bromide, 1:2.5 × 10^7	Histamine dihydrochloride, 1:2.5 × 10^6
A. Metabolic poisons:				
Potassium cyanide	.0004 M	+	+	+
Sodium azide	.0014 M	+	+	+
" iodoacetate	.00004 M	—	+	+
" fluoride	.002 M	+	+	+
" fluoroacetate	.001 M	+	+	+
Dinitrophenol	.0001 M	+	+	+
B. Autonomic drugs:				
Atropine sulfate	1: 7,000	+	—	+
Trasentine	1: 11,000	+	—	+
Hexamethonium bromide	1: 7,000	+	+	+
Mytolon chloride	1: 50,000	+	+	+
Ephedrine	1: 10,000	+	+	+
Epinephrine	1:100,000	+	+	±
Tripelennamine hydrochloride	1:100,000	—	+	—
C. Other substances:				
Ethyl alcohol	1.0%	—	+	+
n-Butyl alcohol	.2%	—	+	+
Urethane	1.0%	—*	+	+
Nupercaine	1:200,000	—*	+	+
Botulinum toxin	(See text)	—	+	±

* Muscle sometimes contracted during subsequent washing with Tyrode's solution.

ulant. Ordinarily each stimulant was applied only once to a particular portion of ileum. All experimental results reported were confirmed with several animals. Additional experiments in which the order of addition of the stimulants was changed showed that the results were independent of this factor. It was found important to remove a stimulating contaminant, apparently ammonium sulfate, from the egg albumin before use. This was done by dialyzing the egg albumin solutions against Tyrode's solution. As the amount of egg albumin necessary for a satisfactory response varied from one animal to another, the level to be used was chosen by first determining the minimum concentration of egg albumin needed for a just perceptible response, and then using 10 times this concentration in the tests with this particular animal.

The experiments with botulinum toxin required a slight modification of the usual technic. The procedure adopted consisted in injecting about 2 x 10^7 units of toxin diluted with Tyrode's solution into the lumen of a short length of ileum tied shut at both ends, and after 30 minutes at room temperature, washing out the toxin, arranging the ileum in the muscle bath, and then performing the tests with antigen or other stimulants. The toxin used was a type D preparation kindly furnished by Dr. George G. Wright, of Camp Detrick, Frederick, Md., and was stated to contain 10^8 mouse units per ml. A recheck of this figure after our experiments had been completed indicated little or no deterioration.

Results. Our first approach to the mechanisms by which antigen stimulates smooth muscle from a sensitized animal was to study the effect of a number of enzyme inhibitors upon the process. A similar approach to related problems has been used by others(11,12,13). Our experiments (Table I) showed that inhibitors that block respiration (cyanide, azide), other oxidative processes (fluoroacetate, dinitrophenol) and glycolysis (fluoride) did not, on short exposure, have an important effect upon the ability of antigen to cause contraction of smooth muscle from sensitized animals. Neither did these substances, under similar conditions, alter contraction due to acetylcholine or histamine. These results led us to a tentative working hypothesis that respiratory and glycolytic processes do not form part of the main chain of events in the Schultz-Dale reaction, although they are undoubtedly essential to the integrity of the tissue and to the recovery process following contraction.

The effect upon the contraction that results from antigen, acetylcholine, and histamine of a number of pharmacodynamic agents known to alter the activity of smooth muscle was also investigated. These results (Table I) generally confirm the numerous reports in the literature, and were useful in interpretation of data described in a later paragraph.

During this work certain observations led us to suspect the existence of a step involving nerve as a part of the reaction:

(1) During storage of ileum that had been removed from animals, ability to be stimulated by antigen was lost sooner than ability to be stimulated by acetylcholine or histamine. This behavior seemed to indicate a longer chain of events in the antigen stimulation. Because of the well-known lability of nerve, this hinted that some nervous element might be involved in the process as a whole.

(2) Iodoacetate (0.0004 *M*) had much the same effect as aging. This also seemed to point to a chain of events involving nerve.

(3) The results obtained with the various pharmacodynamic agents listed in the table were at least consistent with a mechanism involving nerve.

An attempt was therefore made to determine whether substances known to block transmission in nerve were capable of blocking the Schultz-Dale reaction. It was found that ethyl alcohol (1.0%) and *n*-butyl alcohol (0.2%), blocked the Schultz-Dale reaction, but did not affect contraction due to acetylcholine and histamine (Fig. 1, A and B). Nupercaine and urethane (Fig. 2, A) also blocked the contraction resulting from antigen, but when the bath fluid was being replaced with fresh Tyrode's solution, con-

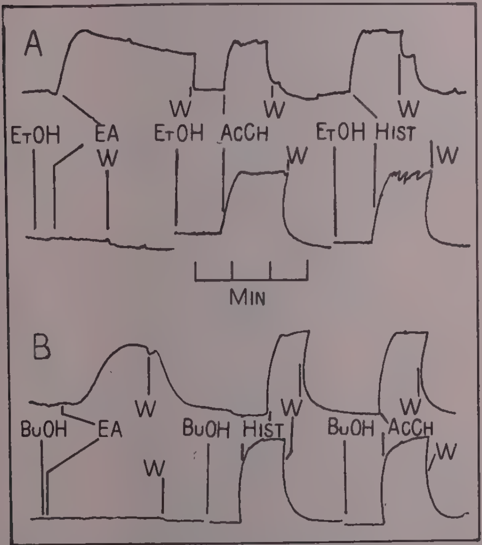

FIG. 1.* A. Effect of ethyl alcohol upon Schultz-Dale reaction. B. Effect of n-butyl alcohol upon Schultz-Dale reaction.

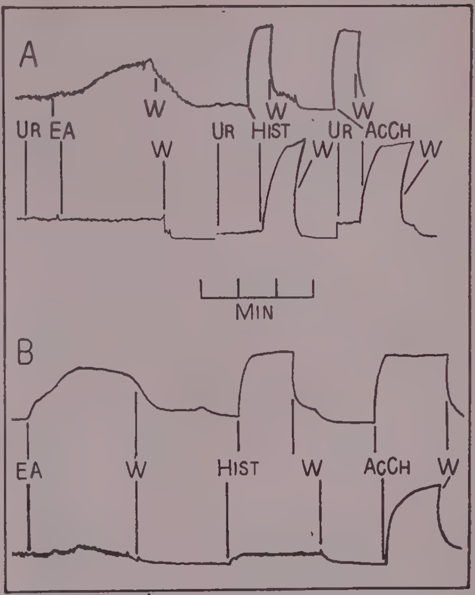

FIG. 2.* A. Effect of urethane upon Schultz-Dale reaction. B. Effect of type D botulinum toxin upon Schultz-Dale reaction. Lower tracing was obtained with ileum treated with botulinum toxin as described in text.

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* Abbreviations used in figures are: EtOH, ethyl alcohol, 1.0%; EA, egg albumin, $1:2.5 \times 10^7$; Hist, histamine dihydrochloride, $1:2.5 \times 10^6$; BuOH, n-butyl alcohol, 0.4%; Ur, urethane, 1.0%; Min, time in min. The recording drum was stopped for 15 min. immediately following each addition of an alcohol or of urethane.

* Abbreviations: See footnote Fig. 1.

traction sometimes took place. Nupercaine and urethane did not alter the effects of acetylcholine and histamine.

These narcotics and anaesthetics are probably not very specific in their action, but seem to be able to block transmission in different kinds of nerves rather unspecifically(14,15), and it seemed desirable to locate the site of action more exactly if possible. The nervous structure involved in stimulation of contraction of the ileum includes preganglionic fibers that enter ganglia of Auerbach's plexus, whence postganglionic fibers lead to nerve endings associated with the smooth muscle. Preliminary evidence that the site of stimulation by antigen may be the postganglionic fibers was the observation (Table I) that the Schultz-Dale reaction remains unchanged in the presence of agents that block at the ganglia, hexamethonium bromide and benzoquinonium (Mytolon).

As Ambache(16,17) and others(18,19,20) have demonstrated, there exists a substance with a very specific paralyzing action upon postganglionic cholinergic fibers, namely, botulinum toxin. Studies with the type D toxin showed clearly that the antigen acts by stimulating the postganglionic fibers, since the toxin blocked the Schultz-Dale reaction, but not stimulation by acetylcholine (Fig. 2, B).

The ability of the toxin to suppress stimulation by histamine was highly variable. Sometimes the toxin suppressed stimulation by histamine rather markedly, (Fig. 2). In other experiments, little suppression was observed. Ambache(17) has recorded similar observations with rabbit ileum. It would appear that either the toxin penetrates to the histamine receptor sites to a different extent in different tissue samples, or that histamine stimulates at more than one kind of site. Our results as a whole seem to indicate that the most likely mechanism for the Schultz-Dale reaction involves stimulation of postganglionic nerve fibers by antigen in the presence of antibody, followed by muscular contraction. Our experiments thus support the theoretical contentions of Campbell(6).

Summary. A study has been made of the effects of a number of substances upon the Schultz-Dale reaction. For the most part, results in the literature concerning the effects of these substances were confirmed. The Schultz-Dale reaction, but not the responses due to histamine and acetylcholine, was blocked by urethane, Nupercaine, lower aliphatic alcohols, and type D botulinum toxin. The results are interpreted to indicate that the Schultz-Dale reaction involves postganglionic cholinergic nerve.

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Synthesis of Diphosphopyridine Nucleotide by the Livers of Mice Bearing Transplantable Tumors.* (22618)

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It was shown(2) that a single injection of acetylpodophyllotoxin-ω-pyridinium chloride into mice bearing Sarcoma 37 induced marked damage in tumors, and that the capacity of the damaged tumors to synthesize diphosphopyridine nucleotide (DPN) from nicotinamide mononucleotide (NMN) was markedly reduced. The livers from these animals, however, exhibited an increased ability to synthesize DPN. When the tumor tissue had only 40% of the activity of control tumors, the livers from the treated mice had an activity of about 130% of the values of the livers of untreated tumor-bearing animals. It should be noted, however, that this capacity to synthesize DPN is still less than that of livers from non-tumor-bearing, untreated animals; in untreated mice the presence of week-old Sarcoma 37 lowered ability to synthesize DPN by about 50%. The synthetic activity with respect to DPN in livers from tumor-bearing mice decreased(2) rapidly to about the fourth day after tumor transplantation; by the sixth day about half of the activity had disappeared. These studies have now been extended to other tumors and tissues.

The present report deals with the DPN-synthetic capacity of livers from animals bearing a variety of transplanted tumors and the extent to which removal of tumor restores the DPN-synthetic function.

Methods and materials. In the first experiment a group of CAF_1 mice [$(BALB/c \times A)F_1$] was inoculated in the tail with implants of Sarcoma 37. The tumor grew slowly; 10 days after implantation, it was approximately 0.6 cm in diameter. Sarcoma 37, implanted in the skin of CAF_1 mice, grew to a diameter of approximately 0.6 cm in 6 days. Lymphoma 1 and Lymphoma 2 also were implanted subcutaneously in CAF_1 mice. Leukemia 1210 was implanted subcutaneously in CDBA hybrids [$(BALB/c \times DBA/2)F_1$] while [$(C_3H \times C_{57}BL/6)F_1$] mice were employed for subcutaneous transplantation of Hepatoma 129. The ascitic form of Leukemia L-4946 was implanted intraperitoneally in $(C_3H \times AKR)F_1$ hybrid mice.§ The rate of synthesis of DPN by liver and tumor homogenates was followed as described previously(2). Blood, drawn directly from the heart and homogenized with distilled water for enzyme determinations, from both tumor-bearing and control animals, was examined in the same way.

Results. Since the livers from mice bearing Sarcoma 37 showed(2) a decreased ability to synthesize DPN from NMN, tumors in the tail were removed, surgically, by cut-

TABLE I. Synthesis of DPN by Livers from Mice Bearing 10-Day-Old Sarcoma 37 in the Tail.

	DPN formed/mg liver N/hr		
	Control mice (no tumor), µmoles	Mice bearing tumor, µmoles	Relative activity, %
♂	.46	.25	54
♀	.46	.31	67
	48 hr after removal of tumor by caudectomy on 10th day		
♂	.49	.46	94
♀	.46	.46	100

Tumor approximately 0.6 cm wide.
Each value is the mean of at least 4 separate livers.

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[§] This tumor arose in Dr. Law's laboratory on 11/27/51 in an AKR female mouse from transplanted thymic tissue. It has been carried as a solid tumor for several generations by Dr. Law, and also has been carried in the ascites form for 90 generations.

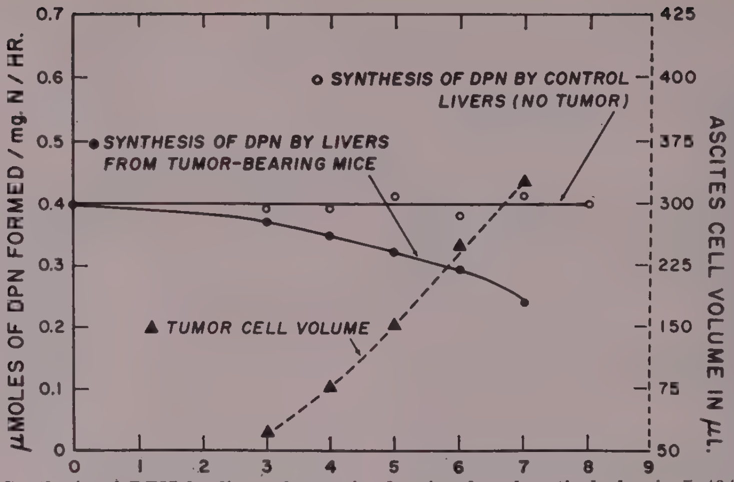

FIG. 1. Synthesis of DPN by livers from mice bearing lymphocytic leukemia L-4946 in the ascites form.

TABLE II. Synthesis of DPN by Livers from Mice Bearing 6-Day-Old Sarcoma 37 in the Skin

	DPN formed/mg liver N/hr		
	Control mice (no tumor), μmoles	Mice bearing tumor, μmoles	Relative activity, %
♂	.48	.31	65
♀	.49	.32	65
	2 days after sham operation	2 days after removal of tumor	
♂	.49	.48	98
♀	.50	.49	98

Tumor approximately 0.6 cm wide.
Each value is the mean of at least 3 separate livers.

ting off the tails to determine whether there would be restoration of the capacity of the livers to synthesize DPN. The findings are summarized in the first 2 tables.

Table I shows that the synthesis of DPN by livers from mice bearing 10-day-old sarcoma 37 in tail was about 60% of the value given by livers from control mice. In all these experiments, at least 4 animals were employed in each group; the results obtained were almost identical within each group and hence only the average values are given. About 48 hours after removal of the tumor on 10th day, the enzyme activity of the livers returned to normal.

The results with livers from mice bearing intradermal tumors are listed in Table II. Sarcoma 37 was implanted in the skin in such a way that, after 6 days, it was banana-shaped, and approximately 0.6 cm wide. Here, too, only 65% of the activity of control livers was obtained. From another group, tumor tissue was removed surgically[‖] on the 6th day as completely as possible (nembutal anesthesia, 80 μg/g), the skin sewed, and 600 units of penicillin administered to minimize infection. Two days later the animals were sacrificed for determination of DPN-synthesizing capacity of their livers. It was found that, after removal of tumor, the enzyme activity of the livers returned to normal. Sham operation in control animals had no effect on the synthesis of DPN by their livers. These experiments were repeated at least 3 times each, with excellent reproducibility; hence only the mean values are given in Table II.

Shacter reported(3) that the glutathione level in livers from mice bearing lymphocytic leukemia L-4946 decreased to about 70% of the levels in control livers. Accordingly, biosynthesis of DPN by the livers from mice bearing this tumor in ascites form was next investigated. Fig. 1 shows the synthesis of DPN by the livers of mice bearing lymphocytic leukemia L-4946 ascites with relation to the volume of tumor cells. As the total volume of the tumor cells[‖] increased, the synthe-

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[‖] By Betty Achinstein of this laboratory.

TABLE III. Synthesis of DPN by Homogenates of Tumor and of Liver.

	Control mice (no tumor)	Tumor-bearing mice	
Tissues*	Synthesis of DPN,† μmoles	Synthesis of DPN,† μmoles	Age of tumors, days
Sarcoma 37	—	.18	6
Liver	.48	.25	
Lymphoma 1	—	.19	15
Liver	.48	.27	
Lymphoma 2	—	.24	15
Liver	.49	.34	
Hepatoma 129	—	.25	15
Liver	.42	.28	
Leukemia 1210	—	.30	6
Liver	.49	.35	
Leukemia L 4946 ascites	—	.16	7
Liver	.41	.24	

* CAF$_1$ [(BALB/c × A) F$_1$] mice were employed in groups for control and bearing Sarcoma 37, Lymphoma 1, and Lymphoma 2. CDBA hybrids [(BALB/c × DBA/$_2$)F$_1$] were used for Leukemia 1210; [C$_3$H × C$_{57}$BL/6)F$_1$] for Hepatoma 129; and C$_3$H × AKR F$_1$ hybrids for Leukemia L 4946.

† Expressed as μmoles of DPN formed/mg N/hr.

sis of DPN by the livers of the animals decreased; 7 days after transplant, the volume of packed tumor cells had increased to 340 μl while the capacity of the livers from these mice to synthesize DPN had decreased from 0.40 to 0.24 μmoles DPN formed, *i.e.*, a drop of 40%.

Table III summarizes the results on synthesis of DPN by livers and tumors from mice bearing Lymphoma 1, Lymphoma 2, Hepatoma 129 and Leukemia 1210. In all these cases, the livers from tumor-bearing mice showed a 30 to 50% lowered capacity to synthesize DPN than livers from control mice. The values on the synthesis of DPN by the tumors such as Leukemia L 4946, Sarcoma 37, Lymphoma 1, Lymphoma 2, Hepatoma 129 and Leukemia 1210 were from 0.16 to 0.30 μmoles DPN formed. In most of the tumors the DPN synthesizing activity was 70% of that of the livers from mice bearing these tumors. The tumors from mice bearing Leukemia 1210 or Hepatoma 129 showed an activity of 86 and 89% of the activity of livers from these animals.

Fig. 2 summarizes capacity of the blood from mice bearing intramuscular Sarcoma 37 to synthesize DPN. Within 24 hours after tumor transplantation, enzyme activity of whole blood from mice receiving the implant dropped to about 50% of the activity of blood from control mice, and remained at this level.

Discussion. The data presented here extend our previous work(*2*) on synthesis of DPN by the livers from mice bearing Sarcoma 37. It was found that the livers from mice bearing various transplantable tumors, such as a Lymphoma, Lymphocytic leukemia or a Hepatoma, also showed this lowered capacity to synthesize DPN. With ascites tumor also, as the tumor cell volume increased the capacity of the liver to synthesize DPN decreased. Surgical removal of the tumor was followed by return of DPN synthetic capacity of the liver to normal. DPN synthesis by blood dropped sharply within 1 day after inoculation of tumor into mice. Preliminary experiments on injection of normal tissues, such as liver, kidney, spleen or heart, into

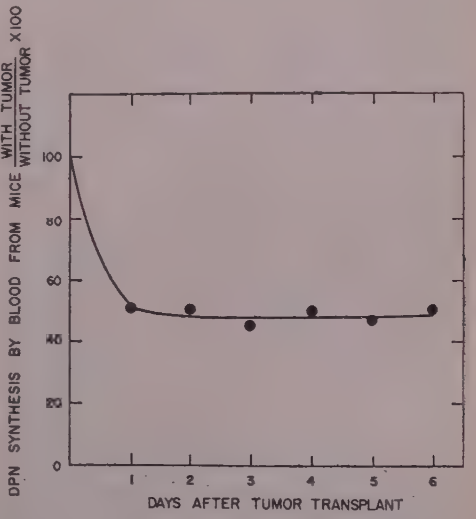

FIG. 2. Synthesis of DPN by blood from mice bearing Sarcoma 37. Range of control values: .123-.129 μmoles of DPN formed per mg N per hr. The data are given in terms of percentages of the control values, *i.e.*,

$$\frac{\text{DPN synthesis by blood from tumor-bearers}}{\text{DPN synthesis by blood from control mice}} \times 100.$$

[‖] We are indebted to Dr. Shacter for furnishing the homogenates of livers from mice bearing L-4946 in the ascites form and also for providing information on the cell volume of the tumor.

mice failed to produce inhibitory effect on DPN-synthesizing enzyme of the blood of these animals. Information is also being gathered on the effect of transplanted tumors on DPN synthesis by tissues other than liver or blood. Growing tumors may exert their influence on DPN synthesis by systemic metabolic competition with other tissues. On the other hand, the thesis that the tumor contains an inhibitor which is transmitted through the blood and which blocks its synthetic capacity to form DPN is also being explored. Search is being made for a humoral factor either in tumor tissue or in tissues from tumor-bearing hosts which may inhibit the synthesis of DPN from NMN.

Summary. 1. Homogenates of liver from mice bearing transplanted tumors (Sarcoma 37, Lymphoma 1, Lymphoma 2, Leukemia L 1210, Hepatoma 129 and Leukemia L-4946) exhibited a drop of 30 to 50% in capacity to synthesize DPN. 2. Surgical removal of Sarcoma 37 restored the DPN-synthesizing capacity of the livers to the level of livers from control mice. 3. Within one day after inoculation of tumor into mice, ability of the blood to synthesize DPN dropped about 50%.

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Proteolytic Enzymes. III. Circulating Esterase Activities after Parenteral Trypsin and Chymotrypsin in Rabbits.* (22619)

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The hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester (TAMe) by trypsin, and of acetyl-L-tyrosine ethyl ester (ATEE) by chymotrypsin was first reported in 1948(1,2). The method used, based on titration of acid liberated by hydrolysis of the ester, was rapid, relatively accurate, and required low enzyme concentrations. We were interested in measuring circulating enzyme activity after parenteral administration of trypsin and chymotrypsin and chose the esterase method because of these advantages.

Materials and methods. Crystalline preparations of trypsin (Armour D-531-157) and chymotrypsin (Armour D-530-165) were employed. The respective hemoglobin activities (3) of these lyophilized preparations were 1.47 and 0.78 μg tyrosine/μg/minute. Rabbit plasma was prepared by mixing 9 parts of blood with 1 part of 3.2% sodium citrate, followed by centrifugation. Imidazole buffer was prepared by adding 1.75 g imidazole to 90 ml of 0.1 *N* HCl. The pH was adjusted to 7.4 and the volume brought to 100 ml with distilled water. This solution was diluted 1:10 with saline for use. TAMe, the substrate for trypsin, was present in the final reaction mixture at a concentration of 0.02 M. For chymotrypsin, ATEE was used in a concentration of 0.006 M.

Esterase determinations were carried out separately at 37°C in a small temperature-controlled water bath. Reaction volumes were constant at 4.0 ml, and mixing was continuous with a magnetic stirrer. All determinations were run at pH 7.4. One ml imidazole buffer was added to the reaction mixture except when plasma was present. Saline was used as the diluent for all reagents. Rate of substrate hydrolysis was measured by determining time required at pH 7.4 for liberation of an amount of acid neutralized by 0.01 ml of 0.1 *N* NaOH.

Results. In vitro studies. In the absence of plasma, reaction velocity was linearly related to enzyme concentration. The reaction rates, expressed in μM acid/min./μg enzyme, were 0.45 for trypsin and 0.52 for chymotrypsin. Successive readings for any one determination showed rate to be constant with time, indicating a zero-order reaction.

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* Preliminary report presented at Fed. Am. Soc. Exp. Biol., Atlantic City, April 1956, (*Fed. Proc.*, 1956, v15, 438).

The inhibiting effect of plasma on esterase activities of the enzymes was determined as follows for both trypsin and chymotrypsin. One part of a saline solution of the enzyme was incubated at 37°C with 5 parts (v/v) of plasma. After a definite time, a 0.6 ml aliquot was transferred to an otherwise complete reaction mixture for determination of esterase activity. Fig. 1 and 2 show the results obtained with increasing levels of trypsin and chymotrypsin, respectively, after a 2-minute incubation between enzyme and plasma. Comparative curves for the enzymes in the absence of plasma are also shown.

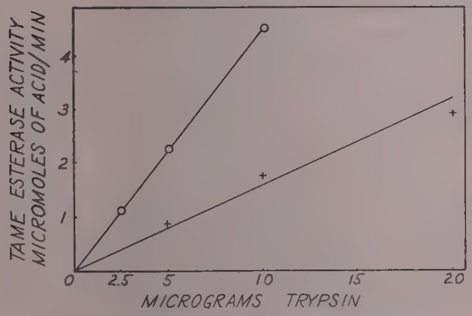

FIG. 1. Relation between trypsin concentration and TAMe esterase activity: (○) in absence of plasma, and (+) in presence of 0.5 ml rabbit plasma.

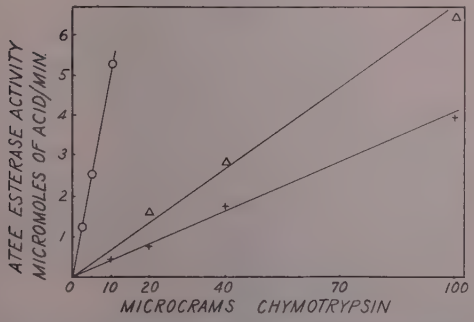

FIG. 2. Relation between chymotrypsin concentration and ATEE esterase activity: (○) in absence of plasma, and (△, +) in presence of 0.5 ml plasma samples from 2 different rabbits.

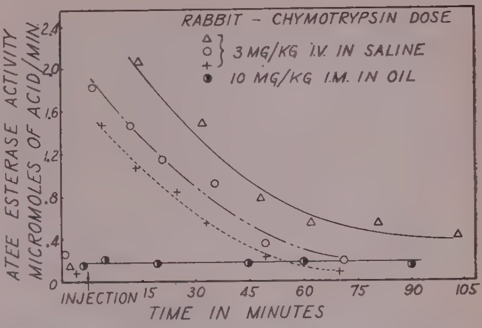

FIG. 3. ATEE esterase activity of 0.5 ml plasma samples withdrawn from rabbits treated parenterally with chymotrypsin.

The percentage inhibition appeared to be nearly constant at the different enzyme concentrations studied with any given plasma sample. However, the inhibition of trypsin varied from 58 to 68% using plasma samples from 6 rabbits. For chymotrypsin, the range was 85 to 93% with plasma samples from 4 rabbits.

The effect of increasing incubation time between enzyme and plasma was also investigated. No significant decrease in esterase activity was found to occur within the 2- to 18-minute interval studied for either enzyme.

In vivo studies. Circulating esterase activity was investigated in rabbits treated with chymotrypsin and trypsin. Chymotrypsin in a concentration of 1 mg/ml in saline was administered intravenously to 3 rabbits at a dose of 3 mg/kg. Blood samples (1.8 ml blood + 0.2 ml 3.2% sodium citrate) were taken by heart puncture before, and at intervals after injection. These were immediately centrifuged for separation of the plasma. Determination of the esterase activity present in 0.5 ml plasma was begun approximately 3 minutes after the heart puncture. Assay conditions were the same as described previously for chymotrypsin.

One rabbit was treated intramuscularly with 10 mg/kg of a commercial preparation of chymotrypsin in oil (Chymar). Circulating esterase levels were measured as described above.

The results for intravenous and intramuscular administration of chymotrypsin are shown in Fig. 3. Intravenous administration re-

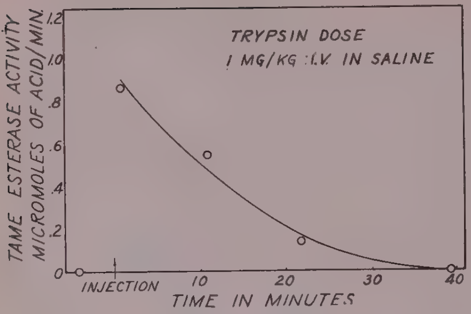

FIG. 4. TAMe esterase activity of 0.5 ml plasma samples withdrawn from a rabbit following an intravenous injection of trypsin.

sulted in a high level of circulating esterase activity which decreased with time. Approximately 50% of the initial activity was lost by 30 minutes after the injection. A small amount of spontaneous activity was measured in plasma samples collected from rabbits prior to injection. An intramuscular injection of a relatively high dose of chymotrypsin in oil did not detectably raise this level of activity.

Circulating esterase activity following administration of trypsin was also investigated. One rabbit was treated intravenously with a dose of 1 mg/kg in a concentration of 0.33 mg/ml saline. One-half ml plasma samples were prepared and assayed for TAMe activity using methods already described. The result is shown in Fig. 4.

Intramuscular administration of trypsin was studied in 3 rabbits. Doses of 1.5 and 7.5 mg/kg in saline were given to 2 rabbits while a third received 7.5 mg/kg in oil. In no instance was any TAMe esterase activity detected in blood samples withdrawn from these animals.

Discussion. In vitro experiments showed the TAMe esterase activity of 2.5 to 20 μg trypsin to be 58 to 68% inhibited by 0.5 ml plasma samples from several rabbits. Parallel experiments with 10 to 100 μg chymotrypsin showed 85 to 93% inhibition of esterase activity. These results indicate low inhibition of esterase activity compared to inhibition of protease activity of these enzymes by plasma reported by other workers(4,5,6,7). Innerfield(8) reported that trypsin administered intramuscularly was clinically effective as an anti-inflammatory agent. This observation has since been confirmed by other workers(9,10). Later, Miechowski and Ercoli(11) working with mice, rats, rabbits and guinea pigs found intramuscular chymotrypsin to be a highly effective anti-inflammatory agent. The mechanism of action whereby these enzymes produce a systemic anti-inflammatory effect is not known. On one hand, it seems possible that the mechanism could involve a prolonged systemic adsorption of the enzymes. Alternatively, the anti-inflammatory effect may result from systemic absorption of local digestion products produced by enzymatic hydrolysis at site of injection. Because of these possibilities we studied circulating trypsin and chymotrypsin levels following injection of these enzymes by the intramuscular route in rabbits. The results showed that no detectable systemic absorption of trypsin or chymotrypsin occurred within the interval studied, even though relatively high doses were used. While this does not prove that systemic absorption of the enzymes is not involved in the anti-inflammatory effect, it must tentatively be considered as evidence against this possibility.

Summary. Inhibition of trypsin and chymotrypsin by plasma has been studied using ester substrates. The amount of inhibition measured was low compared to that reported in the literature using protein substrates, and permitted the measurement of enzyme concentrations in plasma of the order of 10 μg/ml. Intravenous injection of trypsin and chymotrypsin to rabbits resulted in measurable circulating esterase activity, which persisted, in the case of chymotrypsin, up to an hour after injection. No activity was detected following intramuscular trypsin, however, and no detectable increase over a small spontaneous esterase activity was noted following intramuscular chymotrypsin. Thus, it appears that the anti-inflammatory effect noted following intramuscular injection of these enzymes is probably not mediated by their systemic absorption.

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Propagation of Strain L (Earle) Cells in Agitated Fluid Suspension Cultures.* (22620)

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Several workers, using various technics for measuring increases in cell populations, have shown that a characteristic growth curve exists for mammalian tissue cells cultivated *in vitro*(1,2,3,4). As is true with microbial populations, this growth cycle can be separated into 4 major phases: 1) lag phase, 2) logarithmic growth phase, 3) stationary phase, and 4) logarithmic death phase(2,5). The study reported here made use of the rate of change of growing cell populations to demonstrate the effects of inoculum size, pH and serum concentration on strain L mouse fibroblasts in agitated fluid suspension cultures. It was hoped that an analysis of the growth curve would make it possible to establish a more efficient system for cultivation of these cells.

Materials and methods. Strain L mouse fibroblasts(8) were used in this study. The stock strain was grown in stationary cultures on the surface of bottles with a capacity of 250 ml and a surface area of approximately 56 cm^2. Each culture contained 10 ml of a nutrient medium composed of 20% horse serum, 60% modified synthetic solution 199[†] (9), 20% Hanks balanced salt solution, and 100 units per ml each of penicillin and streptomycin. The fluid suspension culture method (6,7) was used in these studies. In this type of culture, 80 ml of medium of the same composition as previously described, and containing the desired number of cells, was placed in a 250 ml Erlenmeyer flask stoppered with a #6 rubber stopper. During incubation such cultures were placed on a New Brunswick rotary shaker, modified to give a speed of 100 rpm. Daily or twice daily, counts were made from a small sample using a hemocytometer. Since the cells in such cultures remain in a homogenous suspension, no treatment was required to disperse the cells prior to making the counts. The pH of samples taken for counting was determined with a Beckman Model G pH meter. For other cultures used as controls, cells were harvested from a stock culture by removing the growth from the wall of the bottle with a rubber scraper. These cells were evenly suspended in the medium by repeated pipetting. A series of replicate cultures was set up with an inoculum of approximately 200,000 cells per ml by pipetting 10 ml of the cell suspension into each of 10-15 bottles, as described for stock cultures. The initial pH was adjusted to approximately 7.4. Two or 3 of these cultures were harvested each day, and cell counts were made using a hemocytometer.

Results. When the logarithm, to the base 2, of the cell number was plotted against time in

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* This work was supported by USPHS Grant No. C2539 (C) from the National Cancer Institute.

[†] Purines and pyrimidines omitted and medium sterilized by autoclaving.

days, typical growth curves resulted in both stationary and in fluid suspension cultures. It could be observed that after a lag period of about 2 to 3 days the cells began to divide at a logarithmic rate. During the lag period pH of the medium dropped from 7.4 to approximately 7.0 in the fluid suspension culture. A value for the rate of change could be calculated in the logarithmic growth phase, using the formula: $R = \frac{\log Nx2 - \log Nx1}{t_2 - t_1}$ (10), where Nx2 is the number of cells at time t_2 and Nx1 the number of cells at time t_1. This value for strain L mouse fibroblasts for both stationary and agitated cultures was shown to be approximately 2.5×10^{-2}. This calculation was based on population change per hour and would correspond to a generation time, for these cells, of about 40 hours. Two generations and sometimes 3 could be observed in the logarithmic growth phase. The apparent maximum cell density for the system described was about 1.5×10^6 to 2×10^6 cells per ml. When cells were removed from a culture in the logarithmic growth phase and inoculated into fresh medium at a pH of 7.4 the culture exhibited a shortened lag period (Fig. 1). Other growth characteristics were not altered. In these experiments, the logarithmic growth phase began when the pH of the medium had fallen to approximately 7. The rate of growth, generation time and maximum cell density appeared to exhibit the same characteristics as previously described. Since pH 7 seemed to be optimal for growth in this system, medium was adjusted to pH 7 by equilibrating with CO_2 prior to the inoculation of cells. When cells growing in the logarithmic phase were inoculated into this medium, no lag was observed, the cells remaining in the logarithmic phase of growth with no further pH adjustment (Fig. 2).

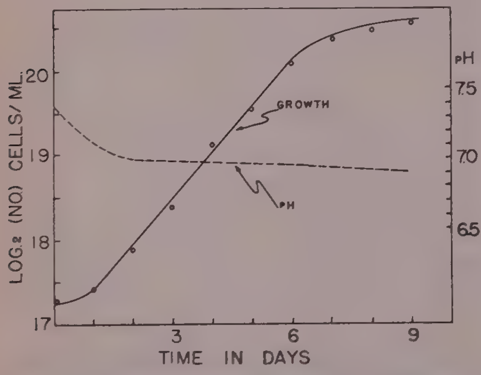

FIG. 1. Growth curve of L strain fibroblasts in shake culture, using cells growing at logarithmic rate as inoculum.

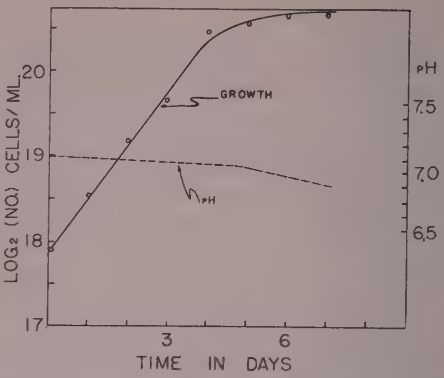

FIG. 2. Growth curve of L strain mouse fibroblasts in shake culture, using cells growing at logarithmic rate as inoculum, and starting at pH 7.

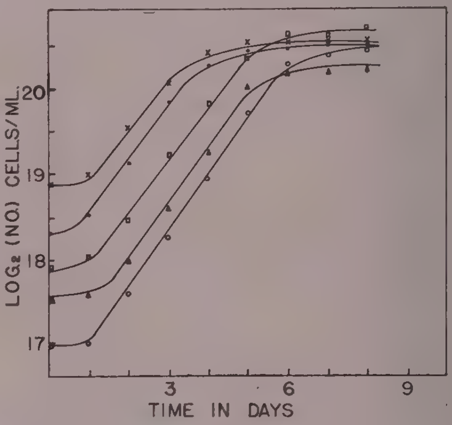

FIG. 3. Growth curves of L strain mouse fibroblasts varying the initial cell inoculum.

With starting inoculums ranging in size from 100,000 to 500,000 cells per ml, rate of growth in the logarithmic phase was independent of the starting inoculum (Fig. 3). An inoculum of 50,000 cells per ml gave the same type of curve. Maximum cell density appeared to be approximately the same for all inoculums tested. It was not possible to es-

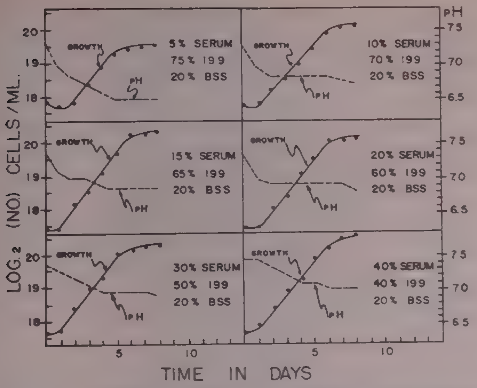

FIG. 4. Growth curves of L strain mouse fibroblasts varying ratio of serum to synthetic medium.

tablish a significant relationship between inoculum size and the length of the lag phase.

With variations of serum concentrations over the range of 5 to 40%, in a complete growth medium, it could be seen (Fig. 4) that rate of growth in the logarithmic phase was essentially independent of the serum concentration. The inoculums for this experiment were taken from a culture growing in a 20% serum medium and in the logarithmic phase of growth. There appeared to be a lower maximum cell density in the cultures containing less serum. It was noted that the apparent optimum pH for growth during the logarithmic phase varied with the concentration of serum in the medium.

Finally, by starting with cells in the logarithmic growth phase and adjusting the transfer interval to a 4 day cycle, conditions of steady state growth were approached. The cells were continually transferred when they were in the logarithmic phase so that cells were available at all times for other studies. When cultures were handled in this manner in 20% serum, no shift in pH below 7 occurred.

These experiments have been repeated, using LLCMl strain mouse fibroblasts(11), with essentially the same results.

Discussion. The development of a growing population of mammalian tissue cells inoculated into an adequate nutrient medium can be divided into a number of successive growth phases that are analogous to microbiological culture cycles and can be described with the nomenclature of Buchanan(5) and Monod(10). Fluid supension cultures of L strain mouse fibroblasts, used in this study, showed growth characteristics analogous to those cited by Rinaldini(2) for chick embryo cells and Graham and Siminovitch(1) for monkey kidney cells. At least 2 distinct advantages of fluid suspension cultures for the study of growth are apparent. When such a culture is established in the logarithmic phase of growth, the cells grow as a uniform, single cell suspension and can be sampled at intervals over a period of time thus eliminating the variation which might exist between replicate cultures. Moreover, cells making up a population in the logarithmic growth phase are more uniform physiologically than cells from other phases of the growth cycle. The complete elimination of the lag period by careful equilibration of pH at the time new medium is added was demonstrated to be possible but not too practical under the conditions of the experiments. A serum concentration of 20% was found to be optimal for L strain cells under the conditions of these experiments. While cultures containing lower serum concentrations showed the same rate of population change during the logarithmic growth phase, the maximal cell density was significantly lower at 5% serum levels and it was more difficult to maintain cultures in the lower concentrations over an extended period of time. Since growth rate and maximal cell density were not appreciably increased at higher serum levels, it was felt that 20% serum represented the most efficient level in terms of utilization of available nutrient materials.

The role of pH in the experiments reported here appeared to be complex. It had been noted in earlier experiments that pH 7.4 was required to assure sticking of L strain cells to a glass substrate. When the pH was allowed to fluctuate significantly above or below this value, many cells failed to attach and remained floating in the medium. Low pH levels, however, while they inhibited cell attachment, did not interfere with the growth of previously attached cells. This fact was not at first appreciated and it was assumed that pH 7.4 was optimal for growth of these cells. When attempts were made to establish fluid suspension cultures at pH 7.4, it was found that the cells had to adjust the medium to a lower pH value before they could begin logarithmic growth. For medium containing 20% serum, this level was pH 7. The pH then remained rather constant during the remainder of the logarithmic growth phase and dropped more rapidly as the cells entered the stationary phase. This phenomenon of adjustment of environmental pH by bacterial populations has been adequately described by Gale(12). The relationship of this adjustment to duration of the lag period was illustrated by the fact that no lag occurred in cultures started at pH 7. It was further noted that one of the possible roles of serum in the medium was to act as a buffering agent. In this regard it was of interest to note that the level at which the pH stabilized during logarithmic growth was directly proportional to the serum concentration.

Summary. The agitated culture has been used in studying the growth cycle of strain L (Earle) mouse fibroblasts. From data available, it is seen that mammalian cells growing *in vitro* display a growth pattern quite similar to that of other microbiological populations. Some points which have been studied and can now be evaluated are the following: 1) the lag period for these cells appeared to be eliminated completely when log-phase cells were used as inoculum in media equilibrated to pH 7, 2) growth rate appeared to be independent of the inoculum size and also independent of serum concentration, 3) generation time for these connective tissue cells, under conditions studied, was approximately 40 hours; 2 generations could usually be observed in these cultures and saturation concentration in some instances reached 2,000,000 cells per ml, 4) steady-state cultivation of tissue cells has been approached. The results indicated that populations could be maintained in such a manner, and this problem will be investigated.

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Equivalence between Vaccinia Particles Counted by Electron Microscopy and Infectious Units of the Virus. (22621)

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In earlier studies on the relation of the number of vaccinia virus particles to the infectious unit variable results were obtained (1-3). The lowest average ratio of particles to infectious units heretofore reported is 4.2:1(3).

Three features of experiments relating number of virus particles to the infectious unit are most important. Clearly, the method of virus titration to obtain the number of infectious units must be highly sensitive and reproducible. Burnet showed that infectivity of vaccinia virus suspensions could be determined by counting the pocks produced by the virus on the chorioallantoic membrane of the embryonated egg(4). Such titrations have the advantage of expressing infectivity in terms of pock-forming units rather than requiring the calculation of a 50% end point. Moreover, conditions have been defined under which such titrations give precise results(5). Secondly, inactivation of virus particles during preparatory procedures should be avoided. This is readily achieved with vaccinia virus because the infectivity of this virus is relatively stable. Thirdly, it is mandatory to distinguish between the virus particles *per se* and fragments of tissue which might be confused with the virus. The electron microscope provides magnification sufficient for positive identification of the characteristic brick-like shape of vaccinia virus and minimizes the possibility of confusing the virus with tissue debris. The present paper reports results obtained when virus particle counts made in the electron microscope were compared to infectivity titrations made on the chorioallantoic membrane of the embryonated egg.

Materials and methods. Virus. Vaccinia virus, initially a calf lymph preparation, obtained from the New York City Department of Health, was passed on the chorioallantoic membrane of the embryonated egg for a total of 8 passages. Passages were made by the inoculation of 0.2 ml containing approximately 1 x 10^4 pock-forming units (PFU) of stock virus onto the chorioallantoic membrane of 12-day-old eggs. After 3 days of incubation, the infected membranes were removed, weighed, and buffered saline containing 5% inactivated normal horse serum was added to make a 10% suspension. Membranes were homogenized in a blendor and the suspension cleared in an International horizontal centrifuge at 1500 rpm for 10 minutes. The opalescent supernate was stored at —60°C.

Vaccinia virus titrations. The method used for titrations of vaccinia virus has been described previously(5,6). In brief, quarter-log dilutions of stock virus were inoculated onto the chorioallantoic membrane of 12-day-old embryonated eggs and pocks were counted after 3 days of incubation. Inocula were diluted so that 3-20 pocks would be produced per membrane over a 10-fold dilution range. In the calculation of the virus titer, the pock counts at each dilution were averaged for groups of 5 membranes. These numbers were then multiplied by their dilution factors, and this figure was converted to PFUs per ml. The standard deviation of pock counts with this technic was approximately 20%.

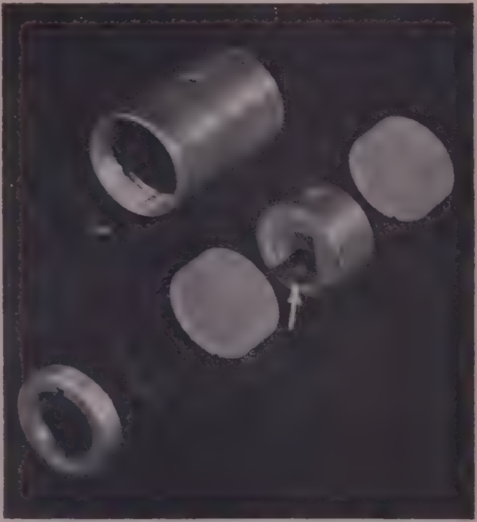

FIG. 1. Centrifugation cell as proposed by Sharp (7) and built by the Specialized Instruments Corp. The present modification includes a stainless steel slide which fits in the trapezoidal chamber and to which are affixed the collodion-coated copper grids (arrow).

Polystyrene latex (PSL). The PSL used in all the following experiments was from Dow Chemical Co., Lot LS-057A. This suspension had a dry weight of 10.1%, particle density of 1.052 and in 577 visual measurements had a diameter of 264 mμ with a standard deviation of 2.3%. The original suspension was diluted 1:20 in double distilled water to make stock suspension No. 1. Over a period of 5 months, 9 dry weight determinations by a micro-gravimetric method gave an average of 5.32 mg/ml. Stock No. 2 represented a 1:100 dilution of No. 1, and Stock No. 3 represented a 1:10,000 dilution of No. 1. On the basis of the dry weight determinations. Stock PSL No. 3 contained 5.25 x 10^7 particles/ml.

Electron microscope preparations. A modification of the method described by Sharp(7) was used. Special cells designed to fit in a 4-hole analytical rotor of a Spinco Model L centrifuge were employed. The essential parts of the cell are shown in Fig. 1 and are similar to those described by Sharp except

TABLE I. Recovery of PSL in Modified Centrifugation Cell.

	Enumeration of PSL* by electron microscopy		
Centrifugation run	Fields counted	Mean count per field†	PSL per ml
1	12	56.7	4.8 × 10^7
2	15	58.5	4.9 "
3	12	61.5	5.1 "
4	21	55.5	4.7 "

Mean No. of PSL particles/ml by count = 4.9 × 10^7

No. of PSL particles/ml determined from dry wt = 5.25 × 10^7

% recovery (dry wt = 100%) = 93.3%

* PSL = Polystyrene latex.

† Counts adjusted to an area of uniform size (see text).

for the bottom metal slide on which collodion-coated electron microscope grids are affixed with glue.* Fluid to be examined was injected into the assembled cells. The precise final volume in the cell was determined gravimetrically. The particles were sedimented directly onto the collodion-coated wire mesh. After the run the grids were removed from the slide, shadowed, and examined in the electron microscope.

Results. Before undertaking studies with the virus, it was important to determine whether, with the cell modification described, accurate particle counts could be obtained after sedimentation. Calculations indicated that sedimentation of PSL would be completed after centrifugation at 6100 g for 23.4 minutes. However, for additional certainty runs were made at 15,750 g for 45 minutes.

In Table I are presented the results of 4 runs with PSL stock suspension No. 3. In each run 12-21 fields were photographed at random at the approximate magnification of 10,000. The precise magnification was determined for each photograph on the basis of the value of 264 mμ for the diameter of PSL particles, and the area of the field photographed was then calculated. It is apparent from the recovery values of PSL sedimented directly onto the collodion-coated screen that the method employed was as accurate as the methods used by Sharp(8). In the present study recovery was 4.9 x 10^7 by count with a suspension which contained 5.25 x 10^7 particles per ml by dry weight. Many fields were examined on the microscope screen to determine distribution of the PSL particles. Distribution appeared to be uniform in all areas. Analyzed statistically, the variation in the individual counts was indicated by the standard deviations of 14, 20, 16, and 54% for runs 1-4 respectively.

Since these results indicated that this simplified method of making electron microscope preparations gave reproducible results with the PSL particles, experiments were designed to adapt this to vaccinia virus suspensions. The 2 stock vaccinia preparations (1 and 2) were titered for infectivity on the chorioallantoic membrane of embryonated eggs. The results are presented in Table II.

Various methods were used in attempts to purify the virus, but although considerable purification could be accomplished as far as obtaining the virus free of debris, loss of virus was great enough to make the methods impractical for present purposes. The following procedure was therefore adopted. The opalescent 10% suspension was diluted 1:100 by quantitively pipetting 1 ml into a 100 ml flask to which was added 1 ml of PSL Stock No. 2 and double distilled water to a volume of 100 ml. This suspension was then used to fill the chambers of the centrifugation cells without further treatment. These preparations contained debris, but due primarily to the unusual shape of vaccinia virus, counts were possible. Moreover, losses of virus particles and inactivation of infectivity were avoided. As with the PSL, the virus preparations were centrifuged at 15,750 g for 45 minutes, a force

TABLE II. Titrations of Vaccinia Virus Preparations on Chorioallantoic Membrane of Embryonated Eggs.

Vaccinia virus	PFU's/ml*	Mean
Prep. 1	3.3 × 10^9	3.2 × 10^9
	3.5 "	
	2.7 "	
Prep. 2	1.4 "	1.4 × 10^9
	2.0 "	
	.8 "	

* Pock-forming units.

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* Scotch masking tape No. 202 was placed in xylol. The solution of adhesive material thus obtained was used.

TABLE III. Numerical Relation between Pock-Forming Units and Virus Particles.

	Enumeration of vaccinia virus particles by electron microscopy				
Vaccinia virus	Fields counted	Mean count per field	Particles per ml	PFU's/ml* by egg titration	Ratio, PFU's/particles
Prep. 1	20	36.4	2.9×10^9	3.2×10^9	1.1
Prep. 2					
Exp. 1	16	13.0	1.0 "	1.4 "	1.4
" 2	23	8.5	.7 "	1.4 "	2.0
				Mean ratio, PFU's/particles = 1.5	

* Pock-forming units per ml.

20 times greater than that needed to sediment the virus, calculated on the basis of a sedimentation constant of 4910 S. PSL was included for precise determination of magnification.

Table III gives the results of these experiments. In all experiments counts were made on photographs rather than on the electron microscope screen. After the grid was placed in the microscope, the first field brought into focus was photographed and thereafter one field of each presenting square was photographed. Concentration of virus particles in the suspensions used was determined on the basis of the number seen per area of the grid and the volume of the fluid in the cell measured gravimetrically (Table III). Concentration of virus particles was also determined on the basis of the ratio of virus to PSL particles in the fields examined and the known number of PSL particles per ml ascertained gravimetrically. The results obtained with the 2 procedures were in good agreement.

It is apparent from the results obtained that the ratio of infectious units to virus particles was very nearly 1:1. Preparation 1

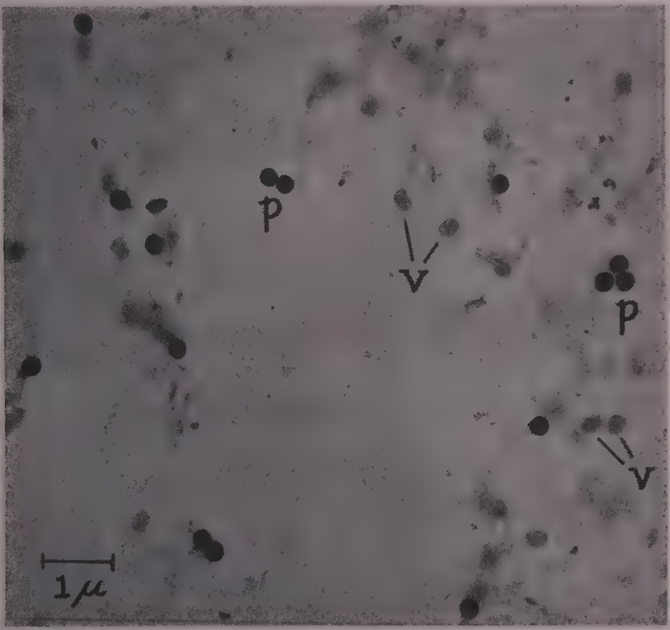

FIG. 2. Vaccinia virus stock diluted 1:100 with distilled water to which PSL was added as an internal control of magnification. The preparation was lightly shadowed with chromium. This field is representative of those obtained with Prep. 2, Exp. 2. P = PSL; V = vaccinia virus.

contained more virus than Preparation 2, both by particle counts and infectivity titrations.

As would be expected, there was considerable variation in the amount of debris from field to field. Fig. 2 is a representative field obtained with Preparation 2. In this experiment virus particles were specially well delineated. Fields containing considerable amounts of debris still left the particles discernible in most instances in this as well as other experiments. No large clumps of virus were found although in some instances groups of 2 or 3 particles were present. No particular association of the particles with debris was noted, and no adsorption of the virus onto the polystyrene balls was observed. Statistical analysis of counts with Preparation 1 showed a standard deviation of 39%. Runs with Preparation 2 gave standard deviations of 23 and 31% respectively for the 2 experiments.

Discussion. The present studies indicate that under the conditions described a single vaccinia virus particle initiates infection on the ectodermal surface of the chorioallantoic membrane of embryonated chicken eggs. There is every probability that under various experimental conditions the ratio of virus particles to infectious unit can change, as indicated by Parker(9). For example, use of a rabbit strain of vaccinia titered for infectivity on the chorioallantoic membrane of the egg may give much higher particle/infectious unit ratios than would the fully egg-adapted strain used in the present experiments. The advantage of the present modification of Sharp's method is mainly that of simplicity and speed. Only a few minutes are required to mount the coated grids on the metal slide, insert this into the cell and make the run. At the end of the run, the grids are ready for shadowing and examination. There is some question as to whether experiments of the type done with vaccinia can be adapted to other animal viruses unless considerable purification of the virus is accomplished first. The shape and density of vaccinia make it difficult to confuse vaccinia virus particles with normal tissue components and make the particles distinguishable even when they are enmeshed in tissue debris. Experiments in this laboratory with influenza viruses have demonstrated the difficulty of working with unpurified suspensions when the virus particle in question is a sphere without other distinguishing characteristics. Work with purified virus suspensions utilizing the "spray-gun" technic has yielded information on the relationship between infectious units and virus particles with bacteriophage(10), influenza virus(11), and poliomyelitis virus(12).

Summary. 1. Studies of the relation between the number of infectious or pock-forming units of vaccinia virus and the number of virus particles indicate a ratio of 1.5:1 under conditions described. 2. A modified centrifugation cell is described with which virus suspensions can be concentrated and the particles then counted by electron microscopy. The virus particles are sedimented directly onto coated electron microscope grids which when removed from the centrifugation cell are ready for shadow-casting and examination. The precision of this method is comparable to that obtained by other technics.

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Complement Fixation by Tuberculopolysaccharide.* (22622)

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The sera of rabbits sensitized by repeated injections with BCG have a high content of antibodies to tuberculoprotein and tuberculopolysaccharide, as can be detected by the precipitin reaction. These sera have been shown in an earlier study(1) also to give high complement-fixation titers with PPD-S, but not with polysaccharide. However, it appeared that the addition of polysaccharide was able to inhibit the complement fixation produced by the PPD-S. It was the object of this investigation to study the mechanism of this inhibition.

Materials and methods. The protein antigen used in this study was PPD-S, a protein fraction isolated from tuberculin culture filtrate after autoclaving. It contained 5.9% polysaccharide. This preparation was adopted as the international standard for purified tuberculins and has been extensively studied (2,3). It proved to be one of the most effective antigens for complement fixation and was used in the previous study(1). Polysaccharide I had been isolated(4) from heated tuberculin filtrate of a human strain of tubercle bacilli (DT), and contained only 0.24% nitrogen. The sera were taken from rabbits used in previous experiments(1). The rabbits had been sensitized (Table I) by repeated injections with heat-killed or live tubercle bacilli or protein fractions. Blood had been taken at frequent intervals and the sera were tested for antibodies by the precipitin reaction. Complement-fixation tests were made by the block titration method in which all dilutions of the antigen were titrated against all dilutions of the serum, using a previously standardized amount of amboceptor and complement and a constant quantity of sheep cells. The sera were inactivated at 56°C for 20 minutes. Every dilution of serum and every dilution of antigen was included in the controls, and in this way it was shown that the antigens used (PPD-S and polysaccharide I) were not anticomplementary, since they by themselves fixed no complement at any dilution. The serum, antigen and complement were incubated in some cases for a half hour at 37°C, and in others kept overnight at 4°C, and then the red blood cell-amboceptor suspension was added. This mixture was then incubated at 37°C for a half hour and placed in the cold room overnight to settle before reading the degree of hemolysis in each tube. The results were plotted, as shown in the curves: each curve encompasses the block of tubes showing no hemolysis, *i.e.*, where complement was fixed by the antigen-antibody system under investigation.

Results. Complement fixation by tuberculoprotein PPD-S and tuberculopolysaccharide I. High complement-fixation titers occurred in anti-BCG sera with the antigen PPD-S (Table I).

The polysaccharide, on the other hand, fixed no complement during incubation at 37°C for a half hour with anti-BCG sera, even though the sera were shown by the precipitin reaction to contain antibodies to polysaccharide. However, if the reaction took place while standing at 4°C overnight, before the amboceptor-blood-cell system (Amb-rbc.) was added, then it was obvious that the polysaccharide did fix considerable complement in those sera containing antibodies to polysaccharide. On the other hand, very little or none was fixed in the case of sera which did not contain antibodies to polysaccharide. This shows that the reaction with polysaccharide was specific. Rice(5) and Eagle(6) both showed that the complement-fixing system becomes more sensitive at low temperature.

The fact that no complement was bound by

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† The technical assistance of Lois Fry, Mabel V. Seibert and Mildred Meekins is gratefully acknowledged.

TABLE I. Complement Fixation and Precipitin Titers.

Rabbits sensitized with	Complement fixation titers						Precipitin titers							
	37°C			4°C			PPD-S				Polys.			
	PPD-S	Polys.	PPD-S + polys.	PPD-S	Polys.	PPD-S + polys.	50	10	5	1	50	10	5	1
BCG (dead)	192	0	0	512	128	24	6+	5+	3+	—	—	3+	4+	6+
	512		0		256	64	6+	5+	3+	±	+	5+	5+	6+
BCG	256	8	48		128		6+	5+	3+	±	+	5+	5+	6+
”	256	0	96				5+	4+	3+	+	2+	3+	4+	3+
”	32		0		0		+	±	±	—	—	—	—	—
” + polys.	64		16		6		5+	3+	3+	—	—	—	—	—
” ”	64		64		0		3+	4+	2+	+	—	—	—	—
” + protein A	64		0		8, 12		4+	2+	±	—	—	—	—	—
Ravenel				48	0	24	3+	+	+	—	—	—	±	—
PPD-S	48		48				4+	2+	+	±	—	—	—	—
Protein A	32		16				5+	4+	3+	2+	—	—	—	—
” A	32		48				5+	5+	4+	3+	—	—	—	—
” C	96		96				3+	+	+	±				

the polysaccharide at 37°C, whereas high titers were given by PPD-S, shows that the antigen in PPD-S responsible for the fixation was not the small amount (5.9%) of polysaccharide present in this antigen, but rather the protein. In fact, it was pointed out(1) that one of the tuberculin proteins, designated as C protein, and which forms a large part of PPD-S, gave as high or higher complement-fixing titers than did PPD-S, and this protein had only 1.1% carbohydrate. The amount of polysaccharide present in PPD-S, and which presumably is the same as polysaccharide I, would be effective merely as an inhibitor to the fixation at 37°C or, at 4°C would slightly increase the titer to the polysaccharide deliberately added to the system. It could not account for the other maximum titer at a lower (1:6400) dilution (curve 2, Fig. 1), which was presumably due to the protein.

It is noteworthy that the range of antigen dilution at which complement fixation was evident was very different for the 2 antigens, PPD-S and polysaccharide. For example, PPD-S fixed complement at 37°C maximally at a dilution of 1:6400 to 1:25,600, whereas polysaccharide fixed complement only at 4°C maximally at a dilution of about 1:220,000 to 1:27,000,000. The two reactions could, therefore, be easily distinguished from each other, as shown in the diagrams of their block titrations (Fig. 1 and 2). A parallel distinction was noted between the ranges of dilution for the precipitin reaction of the protein antigen-antibody system (PPD-S-AAb) and that of the polysaccharide-antigen-antibody system (Polys.-AAb) (Table I). For example, the greatest precipitate occurred with 50 γ PPD-S antigen, whereas with the polysaccharide one γ or less gave the heaviest precipitate, but both of these reactions took place at 37°C. Evidence that they really are different reactions is found in the fact that the characters of the 2 antigen-antibody precipitates were different(1).

Mutual effect of protein and polysaccharide in the complement-fixation reaction. If protein and polysaccharide were mixed in the ratio of 1:7 for use as antigen, the complement-fixation titer to PPD-S in serum containing

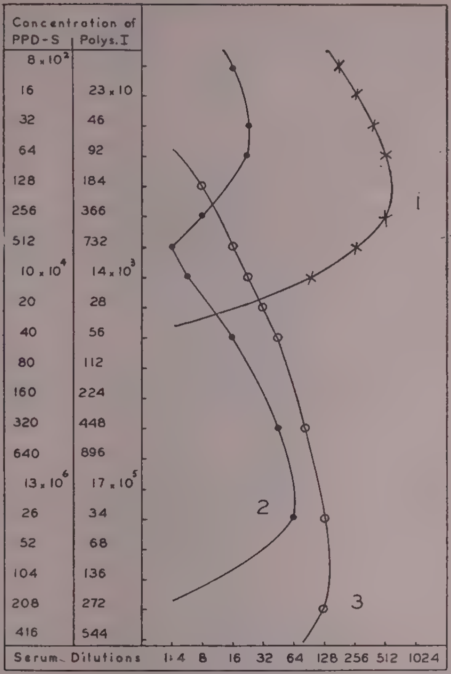

FIG. 1. Block titration diagrams of complement fixation at 4°C by: Curve 1 (X———X) PPD-S antigen-antibody system, curve 2 (●——●) PPD-S + polysaccharide antigen-antibody systems, curve 3 (○———○) polysaccharide antigen-antibody system. Areas encompassed by the curves include tubes showing no hemolysis.

antibodies to both protein and polysaccharide was lower than if the protein alone was used as the antigen, and in many cases it even became zero. This was true if the complement-fixation test was made in the usual way of incubating at 37°C. However, if the fixation took place at 4°C overnight, then it was obvious that both the protein-antigen-antibody system and the polysaccharide-antigen-antibody system fixed complement. Two titer maxima appeared at different antigen dilution ranges (Fig. 1, curve 2). Moreover, the sum of the amounts (indicated by serum titers) of the antibodies to protein, PPD-S, (upper part of curve 2) and to polysaccharide (lower part of curve 2) which fixed all the complement, was equal to the amount of antibody to protein which was required when the protein-antigen-antibody-system was present alone (curve 1).

Mechanism of binding of complement by protein and polysaccharide antigen-antibody systems. To study the difference between the binding of complement by the PPD-S-AAb and the Polys-AAb systems, the followng experiments were performed with the same serum; (1) BCG antiserum, PPD-S and complement were set up in a block titration arrangement in the usual manner, but the first antigen (PPD-S) was added in half the volume normally used. (The proper antigen dilution was in 0.05 ml instead of in 0.1 ml.) The tubes were held overnight at 4°C, then the respective serial dilutions of polysaccharide were added, also in half the usual antigen volume, in order to maintain a constant final volume. In all tubes a ratio of 1:7 of protein antigen to polysaccharide antigen was maintained. The dilutions of each antigen are shown at the left in Fig. 1 and 2. After a

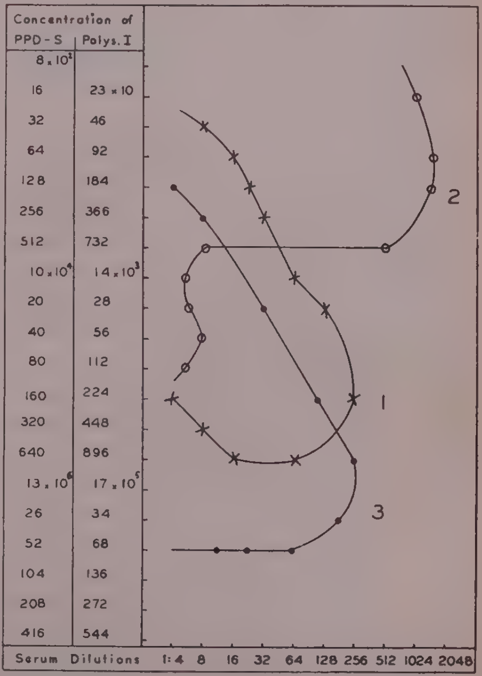

FIG. 2. Block titration diagrams of complement fixation at 4°C by: Curve 1 (X———X) polysaccharide antigen-antibody system alone, curve 2 (○———○) PPD-S added first and then polysaccharide, curve 3 (●———●) polysaccharide first and then PPD-S. Areas encompassed by the curves include tubes showing no hemolysis.

half hour incubation at 37°C the Rbc-Amb. system was added and the mixture was again incubated at 37°C. The resulting block titration diagram, (curve 2, Fig. 2) shows that considerable complement was fixed mainly in the dilution region where the PPD-S-AAb system was effective. A similar technic was used in which polysaccharide was first mixed with BCG antiserum and complement, and PPD-S was added on the following day. In this case (curve 3) considerable complement was also fixed, but this time mainly in the dilution where the Polys-AAb system was effective.

These 2 experiments showed that at 4°C both antigen-antibody systems were able to fix complement in a normal manner, and the system first exposed to the complement was the one effective in fixing it. Therefore, both systems would compete for the same complement if they are present simultaneously.

However, since the polysaccharide appeared to fix no complement at 37°C, but did cause a decrease in the titer given by PPD-S, the interpretation, in the light of the above experiments, might be as follows: The polysaccharide succeeded in holding its share of complement through the "first stage" of the reaction with an avidity which was sufficient to keep it from being fixed by the PPD-S-AAb system, but not sufficient at 37°C to hold it through the second and final stage of fixation, and so the complement was released from this system to the Amb-rbc. system when it was added. The final titer would, therefore, represent only that portion of the complement which had been held by the PPD-S-AAb system, and it would, therefore, appear that the polysaccharide had inhibited the complement fixation.

Summary. 1) High complement fixation titers with PPD-S were obtained with BCG antisera which showed high precipitating antibodies to PPD-S and to tuberculo-polysaccharide. 2) Polysaccharide fixed no complement even in these sera when incubation was carried out at 37°C. However, at 4°C fixation did take place. 3) When polysaccharide was mixed with PPD-S the complement-fixation titer was decreased from that obtained with the PPD-S alone, suggesting inhibition of fixation at 37°C. However, this decrease appeared to be due to fixation by polysaccharide in the first stage, followed by a probable release of the complement to the red-blood-cell-amboceptor system in the second stage.

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Adrenal Cortical Capacity and Cortisol Utilization in Normal and Toxemic Pregnancies.*† (22623)

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It has been demonstrated that the plasma level of 17-hydroxycorticosteroids (17-OHCS) in pregnancy is elevated over normal, non-pregnant values(1-4). No difference in this steroid level was found between normal and toxemic pregnancies(3). Speculation as to the source or sources of the additional 17-OHCS has suggested several possibilities among which two appear probable: an increased rate of production by the maternal adrenal cortex, and faulty utilization by the maternal organism.

Samuels and his coworkers(5,6) have presented evidence for 1) elevation of 17-OHCS in surgery, 2) reduced rate of metabolism of infused cortisol by surgical patients, and by non-surgical patients with liver disease, and 3) correlation between impaired liver function and the magnitude of the rise in plasma 17-OHCS due to surgery. They also believe that there is evidence that liver function is depressed during other types of stress as well as during surgery(6). Hence, they concluded that the elevated levels of 17-OHCS seen in stress are due to both adrenal stimulation and impaired hepatic function(6).

In view of their findings, we decided to investigate the possibility that the increased levels of 17-OHCS in pregnancy are due to both adrenal stimulation and impaired utilization since pregnancy is regarded as a "stressful" situation. We used the test of adrenal cortical capacity or reserve as outlined by Eik-Nes *et al.*(7) to determine whether the stimulus received by the adrenal cortex during pregnancy was maximal. In addition, from the results of this test the functional status of the adrenal cortex might be judged. To determine whether diminished utilization is involved in the production of the high levels of these steroids found in pregnancy, the ability of the pregnant patient to handle infused cortisol was investigated. Both normally pregnant and toxemic pregnant patients were used in this study in the hope that some difference in the production, or utilization, of these steroids under the experimental conditions used might be disclosed, since no difference in these levels for normal and toxemic pregnancies under the influence of endogenous ACTH was found in the previous study(3).

Methods.§ These studies were performed on 39 pregnant patients who were hospitalized and at bed rest. Tables I and III give age, diagnosis, and length of gestation of the patients. The test of adrenal cortical capacity was carried out on 12 patients with normal pregnancy and 13 patients with toxemia of pregnancy. Each of these patients received 40 mg (40 I. U.) of Armour's ACTH in one liter of 5% aqueous dextrose by intravenous drip infusion over a period of 6 or 10 hours. The dose of ACTH employed was higher than that used by Eik-Nes because it was considered possible that the level of endogenous ACTH in the pregnant patient is elevated. If this is true, then the usual dosage of ACTH (25 mg) would be inadequate to produce a physiological response. Blood samples for the determination of 17-OHCS were taken immediately prior to the infusion (zero hour) and at 2, 4, 6, 8, and 10 hours.

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TABLE I. Effect of Intravenously Infused ACTH (40 mg) on Level of Plasma 17-Hydroxycorticosteroids in Normal and Toxemic Pregnancies.

			17-hydroxycorticosteroids in μg/100 ml plasma								
									Max change and time of occurrence		
Patient	Age	Length of gestation, wk	0 hr	2 hr	4 hr	6 hr	8 hr	10 hr	2 hr	4 hr	6 hr
			Normal pregnancies								
7-D	28	40	54.2	107.0	101.6	119.0					64.8
1-F	40	38	54.0	80.8	100.0	101.5					47.5
9-F	20	40	85.4	100.8	100.5	122.2					36.8
8-G	16	36	40.3	68.4	80.0	95.7					55.4
8-K	20	37	58.7	112.9	111.6	126.9					68.2
2-M	20	38	48.4		97.0	105.8					57.4
7-M	20	39	77.5	86.7	109.0	134.0					56.5
1-N	15	40	67.7	101.3	113.8	117.2					49.5
5-N	18	40	42.3	91.6	91.1	80.4			49.3		
9-N	17	40	48.8	78.5	87.4	94.2					45.4
3-O	23	35	61.7	59.4	116.4	107.9				54.7	
4-Y	24	35	49.6	75.7	93.6	108.8	123.9	131.9			59.2
Median		38.5	54.1	86.7	100.3	108.4					55.95
			Toxemic pregnancies								
2-E	18	36	44.2	104.0	109.4	83.3				55.2	
7-E	30	38	59.0	104.3	86.3	74.7			45.3		
5-F	18	36	86.6	101.8	102.9	98.8				16.3	
7-H	20	37	67.6	51.4	77.1	71.1				9.5	
1-I	20	38	72.3	73.0	108.4	118.0					45.7
7-I	18	32	68.7	73.8	73.9	94.4					25.7
1-J	19	34	59.3	94.6	95.1	92.1				35.8	
9-J	22	36	41.9	69.5	90.4	77.0				48.5	
4-K	20	38	28.6	50.1	51.6	60.7					32.1
8-L	21	36	66.1	98.5	102.4	112.9					46.8
4-V	19	42	55.1	83.4	106.9	138.9	117.2	89.0			83.8
8-X	28	31		58.5	72.2	75.7	77.0	81.7			
2-CC	19	32	35.3	70.3	104.2	88.9	105.0	109.4		68.9	
Median		36	59.2	73.8	95.1	88.9	105.0	89.0		42.2	45.7

The test of the metabolism of cortisol was performed on 8 normal and 6 toxemic pregnant patients. Each of these was given 75 mg of cortisol in 250 ml of 5% aqueous dextrose by intravenous drip infusion over a period of one-half hour. Blood samples were drawn immediately prior to the infusion (zero hour) and at 1, 2, 4, 6, and 8 hours after the start of the infusion. The level of plasma 17-OHCS of all of the samples was determined by the method described elsewhere(3). The sulfuric acid blanks recommended by Bayliss and Steinbeck were not used in our determinations since use of these makes it impossible to run duplicates of the samples from normal, non-pregnant subjects. We feel that duplicates are essential; however, we realize that omission of these blanks might produce non-physiological differences in the values for the 2 groups of patients studied.

Results. Adrenal cortical capacity. The data obtained for the levels of 17-OHCS in this test are shown in Table I. Examination of these values shows that a rise occurred in response to exogenous ACTH in all of the patients studied. In the cases of normal pregnancy it is not possible to judge whether the rise was maximal at 6 hours or not, except in the case studied for 10 hours (Y-4) in which the increase was still proceeding. Most of the toxemic pregnant patients showed a less pronounced rise which reached a peak at 4 or 6 hours. The median rise observed at *6 hours* in response to 40 mg of infused ACTH was about 100% in cases with normal pregnancies, and about 50% in those with toxemia of pregnancy. The maximal median rise obtained by Eik-Nes *et al.*(7) in 39 normal, non-pregnant subjects under similar conditions was 300%. Calculations given in Table II, however, show that the rise in normal pregnancy due to the combined effect of exogenous and endogenous

TABLE II. Effect of Endogenous and Exogenous ACTH (40 mg) on Level of Plasma 17-Hydroxycorticosteroids in Normal and Toxemic Pregnancies.

	Endogenous ACTH Median values		Exogenous ACTH Median values		Endogenous ACTH + exogenous ACTH (6 hr) Median values	
	µg/100 ml plasma	% rise	µg/100 ml plasma	% rise	µg/100 ml plasma	% rise
Normal late pregnancy	54.1				108.4	
Non-pregnant control	28.5				28.5*	
Difference	25.6	89.9			79.9	280.5
Normal late pregnancy						
6 hr			108.4			
0 "			54.1			
Difference			54.3	100.2		
Toxemic pregnancy	59.2				88.9	
Non-pregnant control	28.5				28.5*	
Difference	30.7	107.8			60.4	212.0
Toxemic pregnancy						
6 hr			88.9			
0 "			59.2			
Difference			29.7	50.2		

* Endogenous ACTH only.

ACTH compared with our non-pregnant value is equivalent to the rise found by Eik-Nes.[‖] In the toxemic pregnancies this combined rise is about one-fourth lower. In another study of adrenal cortical capacity, Christy *et al.* (8) studied a small number of patients over a period of 4, instead of 6 hours. They reported finding a rise in plasma 17-OHCS in pregnant patients in excess of the rise in normal subjects in response to exogenous ACTH.

In addition, further examination of the data in Table I discloses that whereas 42% of the toxemic pregnant patients may have reached their maximum level at 6 hours after the administration of ACTH, 50% showed the maximum change at 4 hours, and 8% at 2 hours. In contrast, 83% of the normally pregnant patients may not have reached the maximum level at 6 hours, whereas, only 17% showed a maximum change at 4 hours, and none showed it at 2 hours. These data appear to show that the adrenal cortex of the pregnant patient is submaximally stimulated by endogenous ACTH, and that the response to exogenous ACTH is normal in the cases of normal pregnancy and somewhat reduced in toxemic pregnancy.

Comparison of the data obtained by Sandberg *et al.*(5) for the level of plasma 17-OHCS in surgical patients with our data for normally pregnant patients suggests that the stress of surgery produces a somewhat greater rise in the plasma 17-OHCS than does the stress of pregnancy, but that the adrenal cortical reserve of these 2 groups of patients is similar. The toxemic pregnant patients, on the other hand, show a reduced adrenal cortical reserve when compared with normally pregnant patients and surgical patients.

Cortisol metabolism. The data obtained for plasma levels of 17-OHCS in normal and toxemic pregnancy before and after infusion of cortisol are given in Table III. The difference in the zero hour values for the 2 groups is probably due to the smallness of the groups and the difference in duration of pregnancy. The one hour value (½ hour after the end of the infusion) was the highest in both groups of patients. Calculations given in Table IV show that 95% of the exogenous cortisol had been removed from the blood at the end of one hour in both normal and the toxemic pregnancies. It would seem unlikely that the

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[‖] After this manuscript was prepared Migeon *et al.* (*J. Clin. Invest.*, 1956, v35, 488) reported finding that the adrenal cortical capacity of normally pregnant women is equivalent to that of non-pregnant women.

TABLE III. Effect of Intravenously Infused Cortisol (75 mg) on Level of Plasma 17-Hydroxycorticosteroids in Normal and Toxemic Pregnancies.

Patient	Age	Length of gestation, wk	17-hydroxycorticosteroids in μg/100 ml plasma					
			0 hr	1 hr	2 hr	4 hr	6 hr	8 hr
			Normal pregnancies					
9-O	17	37	55.2	117.9	118.1	60.8	69.6	83.7
5-P	20	37	77.2	194.0	150.3	102.9	91.9	88.2
1-Q	19	40	57.4	126.3	124.8	94.5	56.3	61.8
7-Q	19	38	43.4	154.2	143.9	126.9	86.9	89.8
8-S	21	38	72.2	222.5	156.0	121.5	93.7	92.0
4-T	25	38	70.4	173.0	176.2	106.5	88.8	77.2
1-U	17	35	91.2	214.5	149.4	87.0	87.1	69.0
8-U	26	39	65.9	173.2	111.1	91.3	83.0	70.7
Median		38	68.2	173.1	146.7	98.7	87.0	80.5
			Toxemic pregnancies					
3-R	19	32	49.0	164.2	149.2	124.2	88.4	132.0
10-R	34	36	73.6	148.8	101.9	93.9	57.6	48.4
1-X	29	26	24.0	85.0	67.1	65.0	52.2	47.6
5-AA	16	28	10.0	137.0	79.5	59.1	53.5	42.8
1-BB	19	36	19.8	111.5	94.8	56.4		
6-BB	19	32	56.5	142.9	135.6	105.8	65.5	48.5
Median		32	37.0	140.0	98.4	79.5	57.6	48.4

elevated level of plasma 17-OHCS found in pregnancy was due to faulty utilization, since the amount of cortisol removed in the one hour period was more than 100 times the amount of 17-OHCS due to pregnancy (pregnancy level minus non-pregnancy level).

Discussion. The additional rise produced in the plasma levels of 17-OHCS in pregnancy by infused ACTH appears to indicate that the adrenal cortex in both normal and toxemic pregnancy is submaximally stimulated by the endogenous ACTH. In the normally pregnant patient, however, the combined rise due to the exogenous and the endogenous ACTH is similar to that seen in non-pregnant subjects after infusion of ACTH. This suggests that the adrenal cortex is functionally normal.

TABLE IV. Utilization of Cortisol in Normal and Toxemic Pregnancies. Median values of plasma 17-hydroxycorticosteroids.

Time of collection	Normal pregnancies, μg/100 ml	Toxemic pregnancies, μg/100 ml
1 hr	173.1	140.0
0 "	68.2	37.0
Difference	104.9	103.0
	μg/3.2 l* plasma	μg/3.8 l* plasma
Total 17-hydroxycorticosteroids at 1 hr above 0 hr level	3457	3914
Infused cortisol remaining at 1 hr	4.6%	5.2%

* The plasma vol in pregnancy has been found to be greater at 32 wk (toxemic pregnancies) than at 38 wk (normal pregnancies)(9).

In toxemic pregnant patients, although the elevation in these steroid levels under endogenous stimulation does not differ from that of normally pregnant patients, the response to exogenous ACTH is lower and less sustained. Thus, it would appear that the reserve capacity of the adrenal cortex in toxemic pregnancy is reduced. This reduction might be attributed to one, or both, of two factors. If the adrenal cortex is under elevated stimulation during pregnancy, it is possible that reduction in the adrenal reserve in toxemia is due to fatigue of the gland. In addition, and this appears more likely, there may be a shift in the normal ratio of production of gluco- and mineralocorticoids with an increase in the latter. Since the chief mineralocorticoids are not measured by the Porter-Silber reaction, an increase in the plasma mineralocorticoids would not be detected by use of this assay method. Thus the reduction in adrenal cortical reserve found in toxemia of pregnancy may be a reduction in glucocorticoids only. When a chemical method for determination of mineralocorticoids in blood becomes available, this aspect should be investigated. It has been suggested that toxemia of pregnancy is similar in a number of respects to the syndrome of DOCA intoxication(10). It also has been found that the sodium-retaining activity of urine is increased during late pregnancy with the greatest increase found in toxemia of pregnancy(11). These findings appear to lend support to the aforementioned suggestion.

It was not feasible to study the ability of the maternal organism to utilize exogenous cortisol after delivery. Hence, no estimate can be made of the significance of the per cent of exogenous cortisol remaining in the plasma at a given time, except to say that the maternal organism has the ability to use an amount of cortisol greatly in excess of the increment found in 17-OHCS in pregnancy. It would appear, then, that an increased rate of production, rather than a decreased rate of utilization, is responsible for much, or all, of the elevated level of 17-OHCS seen in pregnancy. It is recognized that the data presented herewith do not rule out the possibility that the placenta and, or, the fetal adrenals may contribute to the elevated level of plasma 17-OHCS.

Summary. Adrenal cortical capacity and cortisol utilization were studied in 20 patients with normal pregnancy and 19 patients with toxemia of pregnancy. It was found that the level of plasma 17-OHCS in late pregnancy can be further elevated by the action of exogenous ACTH. The reserve capacity of the adrenal cortex in normal pregnancy is similar to that in non-pregnant subjects; in toxemia of pregnancy it is somewhat reduced. Utilization of infused cortisol in both normal and toxemic pregnancies does not appear to be faulty; thus utilization does not seem to be a factor in the elevated levels of plasma 17-OHCS found in pregnancy.

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The *in vitro* and *in vivo* Effect of Synkavite on *Histoplasma capsulatum*.*
(22624)

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De Saint-Rat and Luteraan(1) demonstrated that 5-hydroxy-2-methyl-1, 4-naphthoquinone in concentrations of 1:50,000 was active, *in vitro*, against *H. capsulatum*. Synkayvite, Roche (tetrasodium 2-methyl-1, 4-naphthohydroquinone diphosphoric acid ester), is a synthetic water-soluble compound of similar basic structure, potent vit. K activity, and relatively low toxicity. These characteristics, together with stability to heat and easy availability, resulted in the selection of this drug for this study.

It was the purpose of the following experi-

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ments to test the potency of Synkayvite as an inhibitory agent against yeast-phase *H. capsulatum, in vitro,* and as a potential chemotherapeutic agent in experimental histoplasmosis in mice. In addition, studies were performed to evaluate the *in vitro* effect of this drug against certain other fungi, pathogenic and nonpathogenic to man.

Materials and methods. The sensitivity of 8 fungi, (Table I), to Synkayvite was determined on brain-heart infusion agar slants containing the following concentrations of Synkayvite in mg per ml: 4.6, 2.3, 1.2, 0.6, 0.3, 0.15, 0.08, 0.04, 0.02 or 0.01. Care was taken to prevent exposure of the Synkayvite to light, and the slants were incubated for 48 hours to test for bacterial contamination. Control media containing no drug were prepared along with an additional control group containing 0.56, 0.28, 0.14, 0.07, 0.04, 0.02, 0.01 or 0.005 mg of phenol per ml of media to correspond with the amount of phenol present as a preservative in the 8 highest concentrations of Synkayvite listed above. All slants were inoculated with 0.3 ml from 3-week-old broth cultures of all of the fungi except *H. capsulatum* where 0.5 ml saline-yeast-phase suspensions from 3-day-old brain-heart infusion agar slants were employed. The inoculated tubes were sealed with parafilm and incubated in the slanted position at optimal temperatures for a period of 1 month. The amount of growth was recorded as 1 to 4 plus or negative, as compared to controls containing no Synkayvite or phenol, and the minimal inhibitory concentrations of both Synkayvite or phenol noted.

TABLE I. *In Vitro* Inhibitory Effect of Synkayvite and Phenol on Fungi.

	Synkayvite		Phenol controls
Fungus	Minimum conc.* for complete inhibition	Phenol conc.* present	Conc.* for partial inhibition
H. capsulatum	.15	.01	.56
N. asteroides	.60	.07	.56
N. braziliensis	.30	.04	.56
T. mentagraphyta	.60	.07	1.12
M. canis	1.20	.14	1.12
E. floccosum	.30	.04	1.12
Aspergillus fumigatus	.30	.04	.28
Scopulariopsis sp.	.30	.04	1.12

* Concentration in mg/ml of brain-heart infusion agar.

Toxicity studies of Synkayvite for 6-week-old normal white male Swiss mice were divided into 2 parts. Part 1 was designed to determine the toxicity of a single intraperitoneal or subcutaneous injection of 0.2 ml of saline containing 0.3 to 0.55 mg of naphthoquinone per g of mouse. Controls received .07 mg of phenol per g in 0.2 ml of saline, a dose comparable to that contained in 0.55 mg of Synkayvite. The second toxicity experiment was designed to test the maximum daily dose of Synkayvite (from .025 to 0.2 mg/g of mouse) which could be tolerated up to 28 consecutive days. Two groups of controls were used: one received phenol in 0.2 ml of saline in concentrations analogous to those contained in the corresponding Synkayvite doses (Table III); the second received 0.2 ml of saline only, daily. The number of deaths and gain or loss of weight of animals in each group were recorded at the end of 28 days.

The effect of Synkayvite therapy in the mice infected with *H. capsulatum* was tested following intraperitoneal inoculation with 35 million yeast-phase organisms in 0.5 ml of mucin(2). At intervals ranging from 3 days to 10 weeks after histoplasma inoculation, daily intraperitoneal injections of Synkayvite were given up to 28 days, whenever possible. All doses of the naphthaquinone were administered in 0.2 ml of saline, and control animals received the greatest amount of phenol in 0.2 ml of saline received by test animals (Table IV).

Results. All of the fungi tested were inhibited completely, *in vitro,* by concentrations of 0.15 to 1.2 mg/ml of Synkayvite (Table I). That the amount of phenol present in these quantities of Synkayvite was not responsible was demonstrated by the fact that considerably more phenol than was present in the Synkayvite was required for even partial inhibition of growth. These observations were particularly striking with *H. capsulatum* in which only 0.15 mg of the naphthaquinone containing only .01 mg of phenol caused complete inhibition of growth while 0.56 mg of phenol alone did not completely inhibit

TABLE II. Effect of Single Doses of Synkayvite in Normal Mice.

Intraper. inoc.			Subcut. inoc.		
Avg wt (g)	Sk. dose*	No. deaths‡	Avg wt (g)	Sk. dose*	No. deaths‡
15	.30	0/6	16	.30	0/4
16	.35	0/6	15	.35	0/4
15	.40	0/6	17	.40	0/4
15	.45	2/6	16	.45	2/4
17	..50	6/6	16	.50	4/4
15	.55	6/6	15	.55	4/4
16	Phenol control†	0/6	17	Phenol control†	0/4

* Sk. dose: Synkayvite in mg/g of animal.

† Control animals received .07 mg of phenol in .2 ml saline/g of mouse.

‡ Numerator = No. of deaths. Denominator = No. of mice.

growth.

Acute toxicity studies in mice revealed that toxic doses of Synkayvite resulted in peripheral vasodilatation, cyanosis, shock, and death 20 minutes to 4 hours after either subcutaneous or peritoneal inoculation. Table II demonstrates that normal mice could tolerate single doses up to 0.40 mg/g weight. No untoward effect was noted in mice receiving .07 mg of phenol per g, an amount equivalent to that contained in 0.6 mg of Synkayvite. Chronic toxicity studies revealed that the mice could tolerate up to 0.15 mg of the naphthaquinone per g of body weight, daily, for 28 days (Table III). When 0.2 mg per g containing .024 mg/g of phenol was used 3 of 10 mice died as did 4 of 10 receiving .024 mg of phenol, alone.

When infected mice were given Synkayvite in doses of .05 to 0.2 mg per g of weight, between the 3rd to 21st day after inoculation with *H. capsulatum*, a markedly accelerated death rate was observed (Table IV). Death would occur within 20 to 240 minutes after a dose of Synkayvite, with signs of shock similar to those observed in the acute toxicity studies above (Group A, Table IV). In mice receiving 0.2 mg/g 3 days after infection, 8 of 10 died after 1 to 3 doses, or within 6 days after histoplasma inoculation. By the 9th day of infection, or after 6 daily doses of 0.2 or 0.1 mg of Synkayvite, 100% mortality was observed, while 6 of 9 receiving .05 mg died. None of the 6 infected controls receiving .024 mg of phenol, the amount equivalent to that contained in 0.2 mg of Synkayvite, and only 16 of 47 (34.4%) untreated controls (Group G) died during this interval.

TABLE III. Effect of Multiple Intraperitoneal Doses of Synkayvite in Normal Mice.

Daily dose (mg)		Avg wt (g)	Avg wt change after 28 days (g)	No. deaths at 28 days§
Sk.*	Phenol†			
.20	.024	17.4	−2.1	3/10
.15	.018	17.0	− .2	0/10
.10	.012	18.1	+1.0	1/10
.05	.006	17.8	− .8	0/10
.025	.003	16.9	+2.1	0/10
.0‡	.024‡	17.7	−3.3	4/10
.0‡	.003‡	16.4	+1.3	0/10

* Expressed as mg/g of mouse.

† As contained in the Synkayvite.

‡ Phenol controls.

§ Numerator = total No. mice. Denominator = total No. deaths.

When therapy was started 7 or 14 days after infection (Table IV, Groups B, C) increased susceptibility to Synkayvite was more marked in that practically 100% mortalities followed 1 to 3 injections of 0.2, 0.1, or .05 mg. This time interval of 7 to 14 days represented the peak death rate in the untreated controls(2-5) (Group G) where 35 of 47 died of specific infection during the interval. However, the rapid fulminating shock-like type of death was noted only in those mice receiving Synkayvite. This rapidly lethal effect was still evident in mice given Synkayvite which still survived 21 days after infection (Group D, Table IV). However, mice surviving 28 days after infection required 7 to 28 injections before this shock-like syndrome could be induced. When mice which had survived infection induced 10 weeks previously were given up to 28 daily injections of Synkayvite, only 1 of 25 reacted unfavorably to the drug (Group F, Table IV). As can be seen through Table IV, mice receiving .024 mg of phenol per g reacted in a manner similar to untreated infected controls.

Discussion. Alcohol-soluble quinones and quinolines have been known to possess antifungal properties and this subject has been reviewed recently by Oster and Golden(6). These studies demonstrate that tetrasodium 2-methyl-1, 4-naphthohydroquinone disphosphoric acid ester in concentrations of 0.6 mg/

TABLE IV. Effect of Synkayvite on Fatality Rate of Mice Infected with 35 Million Yeast-Phase *H. capsulatum.*

Group	No.*	Post inoc., time therapy started	Daily dose, mg/g†	Deaths during 3-day periods after 1st dose					% fatalities in 28 days‡
				1–3	4–6	7–10	11–14	15–28	
A	10	3 days	.20 sk	8	2				100
	13		.10 ”	4	9				100
	9		.05 ”	4	2	3			100
	6		.024 ph	0	0	4	1	1	100
B	8	7 ”	.20 sk	8					100
	9		.10 ”	9					100
	9		.05 ”	8	1				100
	6		.024 ph	1	3	1	0	1	100
C	8	2 wk	.20 sk	8					100
	7		.10 ”	7					100
	7		.05 ”	7					100
	6		.024 ph	0	2	0	0	1	50
D	7	3 ”	.20 sk	7					100
	7		.10 ”	7					100
	7		.05 ”	6	1				100
	6		.024 ph	1	1	0	0	0	33
E	8	4 ”	.20 sk	1	0	2	2	2	88
	8		.10 ”	0	0	1	0	4	63
	8		.05 ”	0	0	0	0	3	38
	6		.024 ph	0	0	1	0	0	17
F	9	10 ”	.20 sk	0	0	0	1	0	11
	8		.10 ”	0	0	0	0	0	0
	8		.05 ”	0	0	0	0	0	0
	6		.024 ph	0	0	0	0	1	17
G	47	Control	None	0	3	13	19	7	89§

* No. of mice chosen from surviving animals for each therapeutic group.

† sk = daily dose of Synkayvite containing phenol; ph = daily dose of phenol equivalent to amt received by test animals receiving highest amt of Synkayvite.

‡ Fatalities occurring in 28 days after therapy was started.

§ None of remaining mice died after 28 days; death from specific infection rarely occurs 30 days post inoculation(2-5).

ml or less will inhibit the growth of 7 of 8 potentially pathogenic fungi tested while 1.2 mg will inhibit the 8th. This effect was particularly evident against *H. capsulatum* where 0.15 mg/ml of medium caused complete inhibition. However, this promising aspect was not evident during *in vivo* studies. In fact, death rates were actually accelerated when previously infected mice received intraperitoneal injection of Synkayvite. Death would occur within 20 to 240 minutes after administration of naphthaquinone with shock-like signs similar to those observed in non-infected mice receiving toxic doses of drug. This effect was particularly prominent during the 7 to 21-day post-infection time interval when peak deaths from specific infection occurred in untreated controls, but was not apparent when mice which had survived infection 10 weeks previously received 28 daily injections of drug. Thus, it is suggested that the inherent toxic properties of Synkayvite are potentiated during the period of acute infection. Since histoplasmosis is a reticulo-endothelial disease and could alter liver function in a patient with disseminated disease, the question arises as to whether the administration of Synkayvite as a vit. K preparation could possibly aggravate the disease process. These results suggest that close observation for any toxic reactions be instituted in any patient with acute histoplasmosis who should, by any chance, receive a naphthoquinone medication.

Conclusions. 1. Tetrasodium 2 methyl-1, 4-naphthohydroquinone disphosphoric acid ester in concentrations of 0.15 mg/ml inhibited completely the *in vitro* growth of *H. capsulatum* while 0.3 to 0.6 mg/ml caused complete inhibition of *Nocardia asteroides, N.*

braziliensis, Trichophyton mentagrophytes, Epidermophyton floccosum, Aspergillus fumigatus, and a species of *Scopulariopsis. Microsporon canis* required 1.2 mg/ml for complete inhibition. 2. Six-week-old white mice could tolerate a single intraperitoneal or subcutaneous inoculation of Synkayvite up to 0.4 mg/g of weight but not 0.45 mg. Mice could also tolerate up to 0.15 mg/g daily for 28 days. 3. Mice infected with *H. capsulatum* could not tolerate non-toxic doses (to normal mice) of Synkayvite. This effect was particularly apparent between 7 to 21 days, but not 10 weeks, after infection. 4. The possibility that this type of medication in acute histoplasmosis could aggravate the disease is suggested.

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Importance of Anemia in Artificially Induced Metabolic Acidosis. (22625)

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(Introduced by John S. Hirschboeck.)

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It has been established that the plasma carbonic acid-sodium bicarbonate buffer group is the important buffer system in overcoming metabolic acidosis induced by accumulation of organic and inorganic acids. On the other hand, while the erythrocyte with its hemoglobin buffers as much as 92% of the endogenous carbonic acid, its effectiveness against fixed acids is uncertain.

In a given clinical situation acidosis and anemia may co-exist as in the anemic phase of glomerulonephritis, an occasional case of diabetic acidosis and the late stage of pyelonephritis. Bland(1) feels that hemoglobin as administered by transfusion of whole blood or red cell mass is fully as important a buffer as bicarbonate. This study was undertaken to determine whether the presence of anemia in itself had any adverse effect on the body's ability to overcome an artificially induced metabolic acidosis.

Materials and methods. Three volunteer patients suffering from severe blood loss anemia due to bleeding peptic ulcer were studied. These patients had no evidence of cardiac, pulmonary, or renal disease. All tests were performed in the postabsorptive state. At 8:00 a. m. the morning of the test, the bladder was catheterized and the catheter left indwelling. Initial blood serum bicarbonate, chloride, sodium, potassium, non-protein nitrogen, total and fractional proteins, and pH (Beckman pH meter) were determined along with red blood count and hemoglobin determination. (Sahli). An aqueous ammonium chloride* solution was infused intravenously at a constant rate so that 200 mEq. of ammonium chloride would be administered in exactly a 4-hour period.

At hourly intervals for the next 8 hours venous blood samples were obtained for pH and bicarbonate determination. At the same time the urinary catheter was opened and the urine collected under oil for determination of pH and total titratable acidity using 0.1 N NaOH and phenol red as an indicator. The pH of whole blood was determined at 25°C by transfer of the sample from a heparinized 5 cc syringe to the Beckman electrode cup and

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* Ammonium chloride 400 mEq./1., furnished through the courtesy of Abbott Laboratories, North Chicago, Ill.

TABLE I. Changes in Blood pH and Blood Bicarbonate in Anemic and Normochromic Phase.

Patient	Hb (g/100 cc)	RBC (million)	Serum	mEq NH_4Cl					After 233, mEq $NaHCO_3$
				0	50	100	150	200	
I	4.75	2.84	CO_2	22.0	19.0	16.9	14.3	14.0	—
			pH	7.52	7.48	7.48	7.42	7.37	—
	13.0	5.42	CO_2	22.9	20.7	19.0	17.3	16.0	—
			pH	7.50	7.43	7.45	7.42	7.40	—
II	6.0	3.22	CO_2	25.0	21.2	19.0	16.9	17.3	30.6
			pH	7.48	7.48	7.42	7.40	7.38	7.58
	14.0	5.36	CO_2	26.4	22.9	19.9	18.6	17.7	28.5
			pH	7.52	7.50	7.50	7.38	7.38	7.58
III	7.5	2.53	CO_2	23.0	19.5	18.2	17.4	18.2	28.5
			pH	7.55	7.44	7.42	7.42	7.41	7.52
	13.0	4.50	CO_2	22.3	20.3	17.7	20.3	17.7	27.5
			pH	7.52	7.45	7.42	7.45	7.38	7.56

reading within a few seconds after transfer. Total serum carbon dioxide-bicarbonate was determined by the method of Van Slyke-Cullen and was expressed as mEq./Liter of sodium bicarbonate.

Red cell mass was given intravenously during the next week until hemoglobin and red count values were at normal levels and the above procedure was repeated exactly as outlined.

The procedure was varied in the last 2 patients so that 233 mEq. of sodium bicarbonate were administered intravenously in one hour at the end of the seventh hour and the procedure continued to determine the effect of infusion of sodium bicarbonate on blood pH, serum bicarbonate, urinary pH, and titratable acidity.

Results. Infusion intravenously with 50 mEq. of ammonium chloride per hour for 4 hours causes a progressive drop in the blood pH to the end of the fourth hour, with a subsequent rise to initial levels at the end of 8 hours. At the same time, the blood bicarbonate falls reaching its lowest level at the end of the fourth hour, rising slightly and continuing at low levels for the remainder of the 8-hour period—the last 4 hours representing a state of compensated metabolic acidosis (Table I). The urinary pH rises and titratable acidity falls during the ammonium chloride infusion; then the urine becomes very acid the last 4 hours and titratable acidity rises.

Repeating the above test on the same patient one week later with red blood count normal and hemoglobin at least 13 g per 100 cc shows essentially no change from the blood and urinary pattern induced in the patient during the period of anemia. The last 4 hours of both procedures represent a state of compensated metabolic acidosis and although in one patient the bicarbonate was 14 mEq. during the anemic phase and 16 mEq. during the normochromic and normocytic phase, these small variations can hardly be considered significant.

The second part of the procedure shows the effect of an infusion of 233 mEq. of sodium bicarbonate during the state of compensated metabolic acidosis induced in the same manner. During the period of anemia there is a prompt rise in blood pH and blood bicarbonate which rapidly goes to and exceeds normal limits during the next 3 hours. One week later following restoration of blood count and hemoglobin the test was performed again. Note identical response to infusion of bicarbonate as during the anemic phase (Table I).

There is a little difference in the blood pH or bicarbonate during infusion with ammonium chloride in the anemic phase as compared with the normochromic phase. Further, the response of this patient to correction of the acidosis with intravenous sodium bicarbonate is identical whether the patient is anemic or has a normal amount of hemoglobin. Table I shows changes in blood pH and blood bicarbonate in patients in the anemic and the normochromic phase during ammonium chloride induced acidosis and cor-

TABLE II. Urine Changes.

	Hb (g/100 cc)		Total*	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	7 hr	After 233, mEq $NaHCO_3$
				—— NH_4Cl infusion ——							
I	4.75	mEq acid excreted	8.76	.78	1.25	1.19	1.00	1.61	.69	2.24	—
		Urine pH		6.22	5.96	5.90	5.72	5.40	5.28	5.10	—
	13.0	mEq acid excreted	8.28	.41	.88	.94	.95	1.53	1.32	2.30	—
		Urine pH		6.04	6.18	6.10	6.00	5.40	5.00	5.00	—
II	6.0	mEq acid excreted	6.45	1.50	.49	.61	.31	1.30	.79	1.45	0
		Urine pH		6.20	6.35	6.10	6.00	5.70	5.20	5.30	7.50
	14.0	mEq acid excreted	5.60	.18	.32	.63	.57	.87	.98	1.05	.21
		Urine pH		7.20	6.75	6.50	6.30	5.83	5.60	5.50	7.38
III	7.5	mEq acid excreted	6.75	1.20	1.12	.86	.92	.99	.78	.88	.36
		Urine pH		6.2	6.1	6.0	5.9	5.7	5.7	5.5	6.9
	13.0	mEq acid excreted	5.60	.87	1.02	.77	.92	.69	.53	.80	.28
		Urine pH		6.4	6.5	6.3	6.1	5.9	5.8	5.8	7.0

* Total titrable acidity of urine during and following administration of NH_4Cl before administration of sodium bicarbonate.

rection with sodium bicarbonate. Note the nearly identical response regardless of hemoglobin levels. The non-protein nitrogen, sodium, potassium and total protein were all within the normal range for this hospital and are essentially unchanged in the anemic and transfused subject.

Discussion. The body response to an acid load would depend upon and involve the (1) base bound bicarbonate, (2) serum protein, (3) the tissues which release base, and (4) the kidney acidification process. While it is possible that the erythrocyte with its base and hemoglobin buffer would normally play a minor role in combatting acidosis induced by NH_4Cl, the value of correction of severe anemia in overcoming metabolic acidosis has to our knowledge not been previously assessed(2). Administration of ammonia chloride to a patient would be equivalent to adding hydrochloric acid since the ammonium is readily converted to urea. In this study the factors which make up the bulk of the total base of the body are held constant. Serum sodium, potassium, bicarbonate and protein concentrations are not appreciably altered by transfusion of the cell mass. The possibility that the plasma volume is expanded making the *total* base greater in anemia has not been excluded. Nevertheless, infusion of large quantities of red cell mass did not appear to offer any advantage in overcoming acidosis. The renal acidification mechanism is not accelerated following transfusion as is reflected in the same low total acid excreted in the urine (Table II). The erythrocyte does not appear significantly to resist acidosis directly or by transport of electrolytes to the kidneys or other tissues during the 8-hour period following onset of infusion of ammonium chloride. It would therefore appear that the height of the erythrocyte count and hemoglobin level of the blood has little, if anything, to do with overcoming the acidosis induced by ammonium chloride. Similarity of the blood and urinary pattern is striking and nearly identical in the same patient when anemic and when having a normal blood count and hemoglobin level. Furthermore, response to correcting compensated metabolic acidosis with sodium bicarbonate is identical whether hemoglobin and red blood count are normal or subnormal.

If these studies can be translated into clinical terms, it is suggested that in the anemias associated with uremic acidosis or diabetic acidosis, correcting blood count and hemoglobin levels would probably have very little effect on the accompanying acidosis. Although it may be important to have the red blood cell count normal because of the increased oxygen carrying capacity of the blood to vital organs, it is questionable whether the increased hemoglobin and red blood cell count would significantly combat the accompanying acidosis.

Conclusion. 1. Patients were made acidotic with intravenous ammonium chloride during a period when they were anemic and again during a period when the red blood count and hemoglobin values were normal. 2. No significant change in range of blood pH, bicarbonate, urinary pH or titratable acidity was demonstrated during and following infused ammonium chloride during the period of anemia as compared with the period of normal red blood count and hemoglobin levels. 3. No difference in response to infusion of sodium bicarbonate during the state of compensated metabolic acidosis was noted when the patients were anemic as compared with a normal red blood count and hemoglobin level. 4. Correction of a severe anemia is not important in overcoming ammonium chloride-induced metabolic acidosis or in the correction of the ammonium chloride-induced metabolic acidosis with sodium bicarbonate.

The authors wish to thank Miss Dorothy Zoeller and Miss Evelyn Warteberg, medical technologists, Milwaukee County General Hospital, for their assistance in performing some of the determinations in this study.

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Serum Transaminase Levels in Experimental Pericardial Injury.* (22626)

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(Introduced by William G. Clark.)

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Because of recent interest in serum glutamic oxalacetic (SGO) transaminase determinations in the differential diagnosis of myocardial infarction, measurements of the level of this enzyme were carried out after experimental production of acute pericarditis.[†]

Method. Thirteen dogs were studied. Pericardial injury was produced in 9 animals, and the remaining 4 animals were used as controls. After the dogs were anesthetized with pentobarbital sodium, the chest was entered by splitting the distal 3 inches of the sternum. The following substances were placed in the pericardial sac: (1) one cc of sterile sand was used in 3 dogs; (2) one cc of sterile talc in one dog; (3) one cc of a virulent culture of alpha streptococci obtained from human blood cultures in 5 dogs; 2 of these were treated with penicillin and streptomycin to reduce the severity of the infection. The pericardial sac was then sutured with 5-0 silk and the chest was closed. The 4 control animals underwent a sham operation but without manipulation or incision of the pericardium. Twelve-lead electrocardiograms and SGO transaminase samples were taken on all dogs before operation and at 24-hour intervals over a period of 5 days after operation. The animals were then autopsied and color photographs were taken of the visceral and parietal pericardia. Tissue sections were obtained from representative areas of the pericardium and myocardium. The severity of pericarditis was estimated at autopsy and was correlated with the color photographs, microscopic pathology, and electrocardiographic findings. The severity of the pericardial changes by electrocardiogram were estimated on the basis of the amount of elevation of the ST segments. One mm elevation of the ST segments was classified as mild, while an elevation of 5 mm or more in multiple leads was classified as severe

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[*] Aided by funds from the Smart Family Foundation.

[†] Reported in preliminary form at the Western Soc. for Clin. Research, Carmel, Calif., Jan. 1956.

TABLE I. Correlation of Severity of Pericardial and Myocardial Injury with SGO Levels.

		Estimation of severity of injury		Serum glutamic oxalacetic transaminase		
Dog No.	Exp. agent	Pericardial	Myocardial	Initial level (units)	Peak level (units)	Peak time after injury (hr)
1	Control			22	113	24
2	"			16	40	22
3	"			26	90	45
4	"			33	128	24
5	Sand	Mild	Mild	30	49	43
6	α Strep*	"	"	24	50	27
7	" *	"	"	20	73	20
8	Sand	Moderate	"	40	61	22
9	α Strep	"	Moderate	20	130	46
10	Sand	Severe	Mild	32	70	21
11	Talc	"	Moderate	52	206	20
12	α Strep	"	"	28	284	48
13	"	"	Severe	26	392	23

* Treated with penicillin and streptomycin.

pericardial change. The extent of myocardial injury was judged from gross and microscopic appearance; that is, the depth to which the muscle showed damage grossly, and the loss of architecture, infiltration of inflammatory cells, loss of cross striations, etc., seen on microscopic study. This was done by 2 independent observers, neither having knowledge of the laboratory data. The agreement between these 2 observers was good although not absolute. When disagreement occurred the slides were reviewed together until a composite estimate was obtained.

The serum samples were analyzed for SGO transaminase activity by the method of La Due, *et al.*(1,2) and correlated with all of the preceding data.

Results. Comparative data are listed in Table I. Inflammatory changes were found to be limited to the heart. In general, the microscopic picture correlated well with the electrocardiographic changes but in a few instances severe inflammatory changes by histologic study were associated with minimal injury patterns in the electrocardiogram. In the 4 control animals, peak SGO transaminase levels ranged from 40 to 128 units. A similar rise in SGO transaminase was observed to follow chest surgery by Nydick, *et al.*(3) and is presumably due to skeletal muscle damage. In the 9 experimental animals, these values were from 49 to 392 units. Peak values were reached in approximately 24 hours in 9 animals and in approximately 48 hours in 4 animals.

Animals in which both pericardial and myocardial changes were mild or moderate, showed peak SGO transaminase levels that were within the control range. Of the 4 animals with severe pericarditis, 3 had SGO transaminase levels which were significantly elevated above control values. Two of these animals showed moderate myocardial damage. The third animal showed severe myocardial damage and a peak SGO transaminase level of 392 units, the highest in the series. In contrast, the peak SGO transaminase level was well within the control range in the animal with severe pericardial but mild myocardial damage.

Discussion. The clinical and electrocardiographic differentiation of acute pericarditis from acute myocardial infarction may at times be very difficult. In such instances, it would be very desirable to have other means of distinguishing between these 2 entities. SGO transaminase levels have been shown to rise with acute myocardial infarction(1,2,4,5,6) and to be proportional to the amount of experimentally produced myocardial damage (7). Normal SGO transaminase levels have been found in experimentally produced pulmonary infarction(8). Sub-epicardial myocardial damage is known to occur in clinical and experimental acute pericarditis and is likely the main, if not the only cause for the electrocardiographic changes seen with this disease(9,10). Although more myocardial damage would be expected to occur with severe than with mild pericarditis, this is not invariable. The ECG is therefore not always reliable in estimating degree of pericardial injury. The data indicate that SGO transaminase levels may rise when acute pericarditis is experimentally produced. Normal SGO transaminase levels were found when the associated myocardial changes were mild, regardless of the severity of pericarditis. The peak levels showed progressive rises with increase in severity of the myocardial changes. The level of SGO transaminase appears to be determined by severity of the associated myocardial damage rather than by pericardial changes *per se*.

The SGO transaminase level, when elevated, does not help in distinguishing between acute pericarditis and myocardial infarction. When the SGO transaminase level remains normal, however, differentiation can be made between these two diseases, and the clinical and electrocardiographic diagnosis of pericarditis confirmed.

Conclusions. 1. Acute pericarditis was experimentally produced in dogs using sand, talc and cultures of alpha streptococci. 2. The peak levels of serum glutamic oxalacetic transaminase were found to be proportional to the severity of sub-epicardial myocardial damage rather than to the severity of pericardial injury. 3. The serum glutamic oxalacetic transaminase levels help to distinguish between acute pericarditis and acute myocardial infarction only when the levels remain normal.

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Occurrence of Bodies within Endoplasmic Reticulum of Ehrlich Ascites Tumor Cells. (22627)

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A previous communication reported the presence of spherical particles inside the endoplasmic reticulum of Ehrlich ascites tumor cells which had been inoculated with anopheles A virus(1,2). A belief that these particles represented the elementary bodies of anopheles A virus was based on the following circumstantial evidence: 1) Consistent size and structure; 2) high opacity in the electron beam after fixation with osmium tetroxide; 3) localized occurrence in large numbers within a certain time interval following inoculation with the virus; 4) absence of similar particles in uninoculated tumor cells or those

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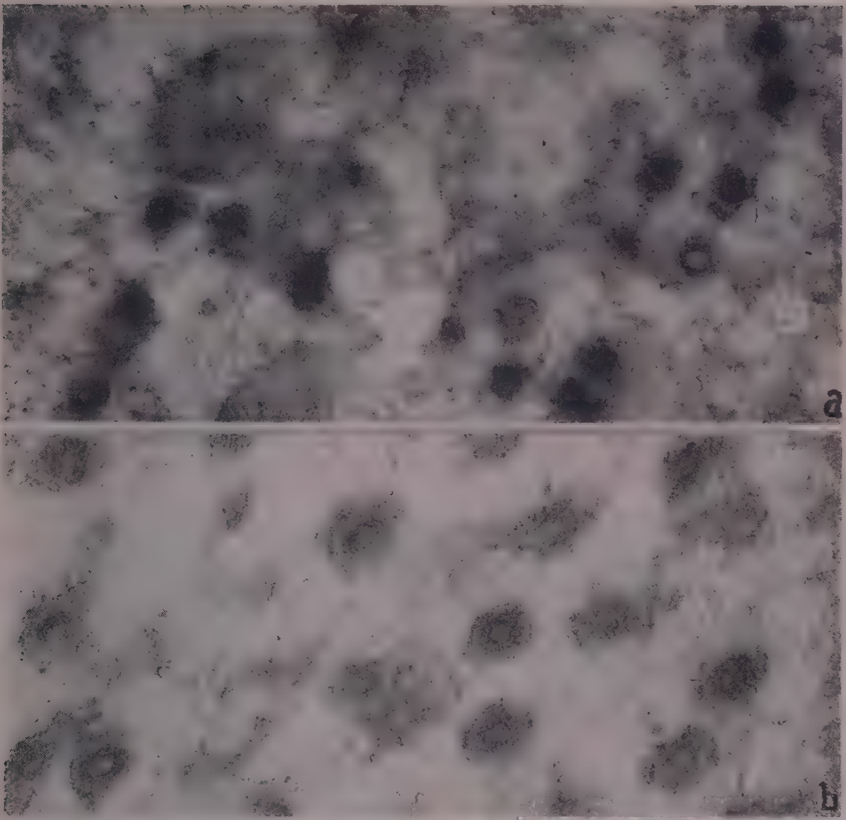

FIG. 1. (a) Thick section (~50 mμ) showing dense bodies in cytoplasm. ICR strain mice inoculated with anopheles A virus(1). × 78,000. (b) Thin section (~30 mμ) showing profiles of the shell structures surrounded by membranes(1). × 100,000.

infected with Mengo, Bunyamwera and Newcastle disease viruses; 5) appearance of such particles in infected chorioallantoic membrane; 6) preliminary results based on ionizing radiation with deuterons indicating a sensitive cross sectional area of the virus not inconsistent with the particles described from electron microscopy.

Further studies have revealed similar particles in the same location of cells not inoculated with anopheles A virus.

Material and methods. The Ehrlich ascites tumor cells were from the same source as those used initially(1,2) *i.e.,* from Dr. T. Hauschka of the Roswell Park Memorial Hospital, Buffalo, N. Y. and were maintained in the ICR (Institute for Cancer Research, Philadelphia) strain of Swiss albino mice at Lederle Laboratories. The same tumor has recently been transferred to the Swiss-Banks strain of mice and specimens from these have also been examined.

Fixing and embedding. Suspensions of the tumor cells were drawn from the abdominal cavity and immediately mixed with 9 volumes of a 1% buffered osmium tetroxide solution (3) brought up to 0.34 osmolar with sucrose. After being fixed for 10 minutes the cells were washed, dehydrated, embedded in N-butyl methacrylate, sectioned with a Porter-Blum (4) microtome and examined in an RCA, model EMU-2B electron microscope.

Observations. Morphology of body. Electron micrograms of particles originally found only in cells inoculated with anopheles A virus are shown in Fig. 1. The particles do not

FIG. 2. A single body found in cell 48 hr after inoculation with neurotropic influenza. ICR strain mice. × 43,000.

usually appear as perfect spheres since sectioning causes appreciable distortion and in some cases natural deviations from a true sphere are evident. Occasional profiles appear horseshoeshaped with the open ends continuous with the wall of the endoplasmic reticulum (Fig. 3). For practical purposes, however, they may be considered as spheres. The thin sections show profiles having a center of low electron density 25 to 30 mμ in diameter surrounded by a complex dense shell 12 to 17 mμ thick, giving an overall diameter of 55 to 65 mμ. Very thin sections at higher magnification indicate that the shell is composed of dense though "hollow" globules 12-14 mμ in diameter.

Occurrence of bodies. Along with every preparation of cells which had been inoculated with virus, control, uninoculated cells were prepared. Twelve such preparations together with 5 in which the ascites cells were

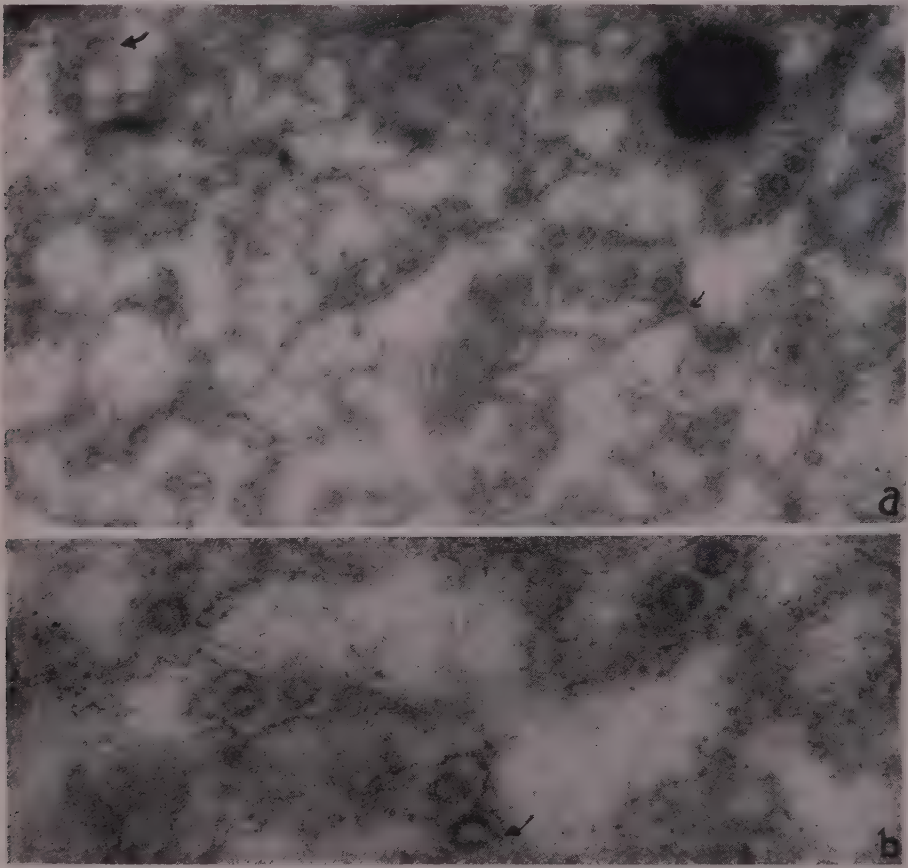

FIG. 3. (a) Numerous bodies inside endoplasmic reticulum of uninoculated control cells. × 43,000. (b) Further enlargement of a portion of (a). Note horseshoe structures (arrows), Swiss-Banks strain mice. × 85,000.

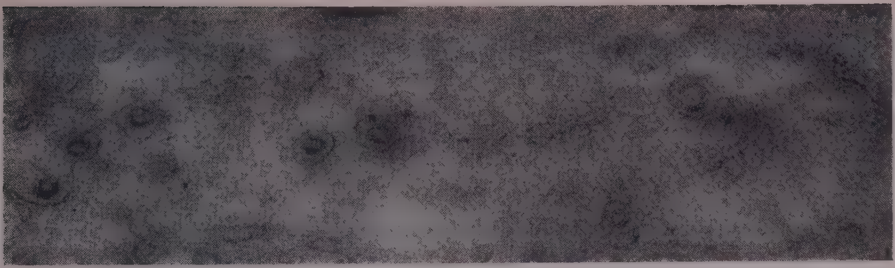

FIG. 4. Appearance of bodies in uninoculated cells carefully assayed for virus. ICR strain mice. × 55,000.

inoculated with viruses other than anopheles A were examined in the electron microscope. The fact that the above-described bodies were not found in these controls does not mean that they were nonexistent, because electron microscopy cannot be used as an assay procedure for, in general, it records only forms that are present in great abundance. The volume of any cell examined in a single section is about 0.1% of the total volume of a cell. However, besides the work in this laboratory there existed the extensive examination of Ehrlich ascites tumor cells at the Sloan-Kettering Institute(5,6) in which none of the above-described bodies were ever seen.

In cells not inoculated with anopheles A virus 2 or 3 of the bodies were first seen in the fall of 1955 in cells inoculated with neurotropic flu virus (Fig. 2). At that time the senior author transferred to Sloan Kettering Institute and started propagating the Ehrlich tumor by transplanting cells from the Lederle-ICR mice at Pearl River to the Swiss-Banks mice at the Sloan Kettering Institute. After 26 passages in the latter mice cells were found to contain large numbers of the same type of structures located within the endoplasmic reticulum as were initially found only in cells inoculated with anopheles A virus (Fig. 3). A recent preparation of control cells directly from Lederle Laboratories also now contains the bodies in every section examined (Fig. 4). These latter cells have been exhaustively tested by the Viral and Rickettsial Division of Lederle for the presence of virus with only negative results.

These bodies have also been identified in ascites tumor cells in other laboratories(7, 8).

Summary. Dense, spherical, shell-structured bodies, measuring about 60 mμ in diameter, which were originally associated only with anopheles A virus have now been found in uninoculated Ehrlich ascites tumor cells. These bodies are found in the cytoplasm within the lumin of the endoplasmic reticulum and in some instances they seem to be attached to or to be formed from the wall of the endoplasmic reticulum tubule.

This work was supported by the Lillia Babbitt Hyde Foundation and the Research Division of American Cyanamid Co., Pearl River, N. Y.

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Studies in Pertussis Immunity. III. Immunization of Children with Live and Killed Vaccine.* (22628)

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The immunity induced by killed pertussis vaccine is known to be less durable and less complete than that induced by an attack of the disease. It seemed reasonable to attribute this difference to some alteration of the antigen brought about by the procedures used to kill the organisms, and to attempt to secure a more complete and durable immunity by vaccinating with live organisms. The relative effectiveness of live and killed vaccines has previously been compared by means of the mouse intracerebral challenge test(1). It was found that live vaccine gave better protection to mice than did killed preparations ($p = 1\%—0.1\%$). In extending this study to the vaccination of children, a method of assaying immunity was needed. Since it was not possible to determine this by direct exposure, it was decided to compare the effectiveness of these two vaccination procedures by a test for passive protection in mice. Groups of subjects, injected with live and killed vaccine respectively, were bled at appropriate intervals. The serum was injected intraperitoneally into mice which were then challenged with live *H. pertussis* by the intranasal route (2). This test has shown good correlation with immunity developing after the disease. Known immune individuals and adults (who are presumably immune) respond to a booster injection with a sharp increase in mouse protective antibody; known susceptibles consistently fail to show any rise in protective power of their sera under these conditions(3).

Using this test we have not been able to demonstrate superiority of the live vaccine in children, but some evidence of a difference in response to the live and killed vaccine was found.

Observations have also been made of the development of antitoxin, and of antibacterial immunity as measured by agglutination, indices which may be regarded as less significant than mouse protection.

Methods and materials. A. Subjects. Half of 40 mentally defective children at the Willowbrook State School were given the live vaccine, the other half the killed vaccine. Age range was from 9 months to 11 years, for the most part between 18 months and 4 years. These children were selected after preliminary testing of their sera showed that they possessed neither circulating antibacterial agglutinins nor passive protection for mice in the intranasal challenge test.

B. Preparation of vaccines. H. pertussis strain #18323 was grown in Cohen and Wheeler fluid medium(4), and the 72 hr growth harvested and suspended in phosphate buffer (0.02 M—pH 7). The cells were washed once with the buffer, then resuspended to a concentration of 20 billion organisms/ml. The cells thus suspended in phosphate buffer were used as the live vaccine preparation. It was used for inoculation within 4 hours of harvesting of the cells.

The killed vaccine was prepared by adding 0.001% merthiolate to the above preparation and storing in the refrigerator; the cells were usually nonviable after 4 weeks and non-toxic after 2-3 months. One small group received the killed vaccine after one month, when the bacilli were no longer viable although the vaccine was still somewhat toxic, in order to use aliquots of the same cells for both the live and killed vaccine groups. As immunizations were spaced at monthly intervals, bacilli of the previous month's live vaccine were used the following month for inoculation of the killed vaccine group.

C. Scheme of immunization and bleeding. The vaccines were divided into 4 doses of 10 billion, 20 billion, 20 billion, and 20 billion

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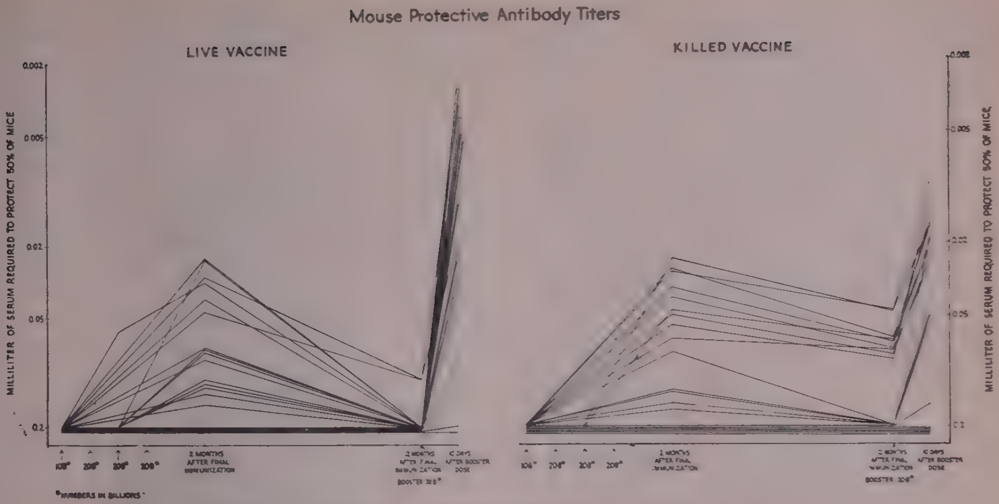

FIG. 1. Response of children to immunization with *H. pertussis* vaccine.

organisms (0.5 ml, 1 ml, 1 ml and 1 ml) and injected at monthly intervals. Injections were given deep into the deltoid muscle of older children or the buttock of the younger ones, alternate sides being used. Blood was drawn before the first inoculation, 4 weeks after the second, and 2 months after the final injection. After 12 months had elapsed, a booster dose of 20 billion organisms in 1 ml volume of a heterologous killed vaccine was administered to both groups of children. Blood was drawn at this time and again 10 days later.

D. Methods of assay. The mouse protection tests and antibacterial agglutination titers were performed by methods previously described(5). Two methods of titration of antitoxin were used. The sera of individuals in each group were tested against 2-4 S.D./50 of pertussal toxin intradermally in rabbits (6). Pools were made of sera from each vaccine group and tested against 2-4 L.D./50 of toxin intraperitoneally in mice(7). Whenever possible the sera of individuals from both groups were assayed together, all sera of each individual being tested at one time.

E. Reactions to Live and Killed Vaccine. Children given the killed vaccine preparation developed neither local nor systemic reactions of any note; there was no evidence of local tenderness, erythema, or induration, and there were no temperatures recorded above 101°F. Among the 20 children given the live preparation, 5 developed fever between 102-103°F. which could be attributed to the inoculation of the vaccine. Three of these children showed a similar rise of temperature after the second inoculation; thereafter, temperature rises were less marked. Local reactions developed in 7 of the 20 children, 3 moderately severe and 4 severe. The remaining 13 children either did not develop local reactions or developed only a very small hard nodule which could be palpated deep in the muscle. The 3 reactions classified as moderately severe were those showing erythema and induration up to 2-4 cm in diameter; erythema faded rapidly and induration disappeared within 7 days. Severe reactions were characterized by the presence of erythema and induration up to 4-6 cm in diameter. These reactions often persisted for longer than one week and occasionally for 3 weeks. These children appeared to develop areas of sub-surface necrosis, but none of the areas showed actual ulceration. One child developed this type of reaction after each of the 4 injections. Another developed a severe reaction after each of the first 3 injections. The remaining 2 children showed such reactions only after the first injection.

Results. A. *Mouse protective antibody*

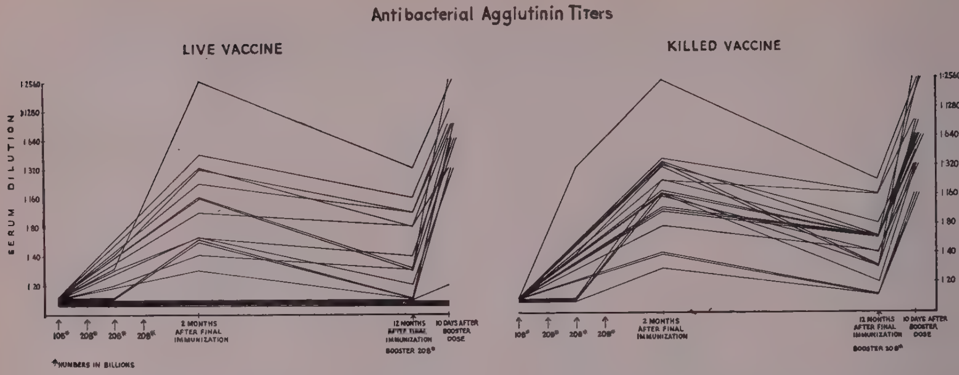

FIG. 2. Response of children to immunization with *H. pertussis* vaccine.

titers. Fig. 1 shows the results of the passive protection tests in mice challenged intranasally. *Initial response:* In those who responded to primary immunization the titers that developed ranged from a P.D./50 of 0.15 ml to 0.024 ml in the live vaccine group and from 0.16 ml to 0.024 ml in the killed vaccine group. Five of the 20 children who were given live vaccine did not develop circulating mouse protective antibodies. The sera of 6 of the 20 children given killed vaccine also showed no rise in this antibody. Over a 12 month period the levels of circulating mouse protective antibody among the children given live vaccine dropped markedly and were still measurable in only 2 individuals. The same was not true of those receiving the killed vaccine. The children in this group who had developed substantial protective titers after immunization retained these titers at moderate levels at the end of the 12 month period. *Booster response:* A booster injection was given one year after the primary immunization had been completed. In response to this booster injection, 10 of the 20 children given the live vaccine developed a 20 to 50-fold or greater increase in mouse protective antibody titer. Six of the children showed a 4 to 10-fold increase. The remaining 4 children showing little or no increase after the booster injection were those who had failed to respond to primary vaccination; the fifth child who had not developed measurable antibodies did show a significant booster response. Fourteen out of 20 children given the killed vaccine responded with increases in titers of roughly 4 to 10-fold; the remaining 6 children who failed to respond had originally failed to produce mouse protective antibody after primary immunization.

B. Antibacterial agglutinin and antitoxin titers. Fig. 2 shows antibacterial agglutinin titers developed by the children immunized with the 2 vaccines. Essentially there were no significant differences in the results. After primary immunization 6 of the children given the live vaccine did not develop agglutinins and only one of these children showed a slight response to the booster injection. Although all 20 individuals given killed vaccine showed increases in titer after immunization, responses in a few instances were low. On the whole, both groups showed a comparable response to the booster injection. The majority of titers after primary immunization were in the range of 1:160-1:320 and from 1:640-1:1280 after the booster injection. Neutralization of pertussal toxin was not demonstrated with sera of either vaccine group.

Discussion. The results of this study give no clear-cut answer as to the merits of the 2 types of vaccine. The number of subjects who responded poorly by any criterion is more than would have been anticipated and raises some question as to whether mental defectives as a group are as readily immunized as the population at large. We also cannot explain the apparently different response to the live and killed vaccine—the more rapid loss of mouse protective antibody titer and the greater booster response seen in the case of the live vaccine. It is unfortunate that an opportunity to challenge the immunity of the two groups did not present itself. To date—some 2 years after immunization was completed—no exposure to whooping cough has occurred.

Summary. 1. The relative effectiveness of live and killed *H. pertussis* vaccine has been studied in children. 2. By means of a passive mouse protection test, using the intranasal route of challenge, a more rapid loss of antibody after primary immunization and a greater booster response was observed with live vaccine. 3. Severe local reactions occurred in some children given the live vaccine.

We gratefully acknowledge the cooperation of Dr. Harold H. Berman and Dr. Martin Lazar of the Willowbrook State School in planning and carrying out this study. We wish to thank Dr. Robert Ward and Dr. L. Emmett Holt, Jr. of this department for their continued help and encouragement. Loretta Brown gave excellent technical assistance.

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Studies in Pertussis Immunity: IV. Booster Response in Man Induced by Various Fractions of *H. pertussis*.* (22629)

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It has previously been reported(1) that sera of children convalescing from whooping cough show a consistent rise in mouse protective antibody. Similarly it has been found that a high percentage of adults respond to a booster injection of killed pertussis organisms with a sharp rise in this same antibody. The present report deals with the ability of various fractions of the *H. pertussis* bacillus to induce this recall response. Four antigens or fractions have been studied: 1) the agglutinogen of Smolens and Mudd(2), 2) the protective antigen of Pillemer(3), 3) the hemagglutinin of Keogh and North(4), and 4) the soluble fraction of Pennell and Thiele(5). Whole cell vaccine was used as the control booster stimulus.

The study was carried out in a group of 100 adults at the Willowbrook State School. Each fraction was tested in 20 individuals and 20 were injected with the control killed vaccine. Blood was drawn prior to intramuscular inoculation of the test booster dose and again 10 days later. The sera were tested for the presence of antibacterial agglutinins as well as for their ability to protect mice against intranasal inoculation with lethal doses of *H. pertussis*.

Methods and materials. A. *Preparation of the fractions.* *H. pertussis* strain #18323 (Kendrick) was used for the fractions prepared in this laboratory. The organisms were grown in Cohen and Wheeler fluid media(6) and collected after 72 hrs incubation. *Agglutinogen* was prepared by the acid extraction method of Smolens and Mudd(2). The final preparation assayed at 1000 agglutinin absorbing units/ml; 250 or 500 units in 0.5 ml was used as the booster dose. This prepa-

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* This study was supported by a grant from the U. S. Public Health Service, Dept. of Health, Education and Welfare.

TABLE I. Agglutinin Response of Adults to a Booster Dose of Various Pertussis Antigens.

	Agglutinogen (Smolens and Mudd)	Soluble antigen (Pennell and Thiele)	Intracerebral protective antigen (Pillemer)	Whole cell vaccine	Hemagglutinin (Masry)
No. of individuals showing response	19	18	13	12	7
Total No. of individuals	20	20	20	20	20
% of response	95	90	65	60	35

ration contained <2 E.D./50 ml when tested by the intracerebral potency test(7). *Pillemer's protective antigen*: Lot #134L32, supplied through the courtesy of Dr. H. D. Piersma of the Lederle Laboratories, did not absorb agglutinins (<25 units/ml) and assayed at 91 E.D./50/ml in this laboratory when tested for intracerebral potency. The product was labeled as containing 26 protective units/ml(8). Approximately 23 E.D./50 or 6.5 protective units in 0.5 ml was used to test for booster action. Protective Antigen was also prepared in this laboratory from strain #18323 by a method similar to that described by Pillemer(3). It was not as potent as Pillemer's preparation containing only 25 E.D./50/ml. It differed from Pillemer's own antigen in that it showed agglutinin absorbing activity. The product was concentrated by centrifuging down the stroma and resuspending it in a smaller volume of saline; 20 E.D./50 in 0.5 ml was used for the booster tests.

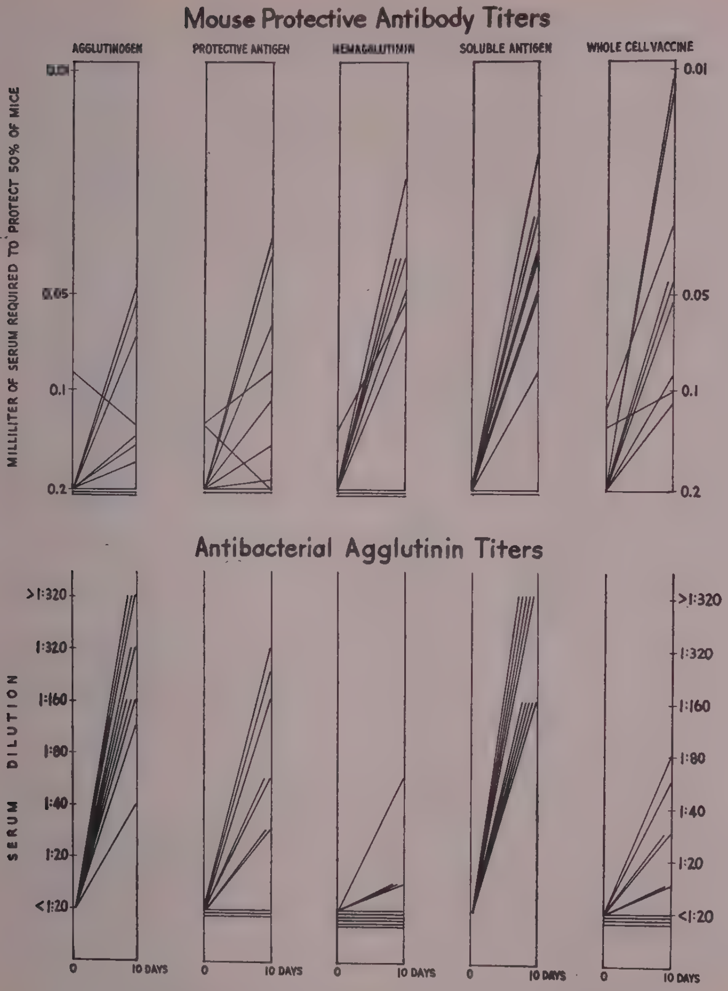
Hemagglutinin Antigen was prepared by extraction #18323 cells with molar sodium acetate for 24 hours at 37°C(9). The hemagglutinin present in the crude extracts was then adsorbed on to stroma by incubating at 37°C for one hour in the waterbath; the stroma was washed several times with saline and finally resuspended in saline. The final product did not absorb agglutinins (<25 units/ml), and did not protect against intracerebral infection (<5 E.D./50/ml). It contained 40 hemagglutinin units/ml; 20 units in 0.5 ml served as the booster dose.

Soluble Antigen was obtained through the courtesy of Dr. W. F. Verwey of Sharp and Dohme Laboratories. Lot #1148-4B was used in these experiments. It had been prepared by the method of Pennell and Thiele (5) and was an alum precipitated product. This preparation contained 1000 agglutinin absorbing units/ml and 71 E.D./50/ml by the intracerebral potency test when assayed in this laboratory; 250 or 500 units of agglutinogen and 17 or 35 E.D./50 in 0.5 ml were used as the booster stimuli. Whole cell vaccine was prepared from *H. pertussis* strain #18323 and suspended in phosphate buffer (0.02 M—pH7) after once washing the cells with the buffer Two different lots were used. They contained 200 agglutinin absorbing units/ml each and assayed at 50 E.D./50/ml and 81 E.D./50/ml respectively. 100 units of agglutinogen and 25 or 40 E.D./50 in 0.5 ml were used as the booster dose.

B. *Assay of the response to the booster dose.* Determination of the antibacterial agglutinin titers and the intranasal passive protection tests were performed by methods previously described(1).

Results. A. *Booster response in adults as measured by rises in antibacterial agglutinin titers.* Table I shows numbers of individuals in each of the 5 groups developing increases in agglutinins. The best responses were obtained with agglutinogen and soluble antigen complex. Hemagglutinin gave the poorest response.

B. *Booster response in adults as measured by mouse protection tests.* Mouse protective antibody was determined in 10 of the 20 subjects studied in each group. The labor involved in these tests precluded studying the entire group. The sera tested were those which had shown the greatest response in antibacterial agglutinins. Considerable discrepancies exist between the results of agglutination and mouse protection tests (Fig. 1). The soluble antigen gave an excellent response in both tests. However, the agglutinogen which gave


Mouse Protective Antibody Titers

Antibacterial Agglutinin Titers

FIG. 1. Booster response of man to fractions of *H. pertussis.*

excellent agglutinin titers performed poorly when tested for mouse protection. Conversely, the hemagglutinin, a poor agglutinin producer induced a satisfactory mouse protective antibody response. Results with the intracerebral protective antigen were not striking when measured by either test. Whole cell vaccine which served as the control showed greater increases in mouse protection than in agglutinins. Where 2 different levels or 2 different preparations of a given antigen were used, there was no difference in the response observed.

Discussion. When passive protection in mice against intranasal challenge was used as the index of booster response, only the soluble antigen and hemagglutinin gave results comparable to whole cell vaccine. The complex nature of the soluble antigen fraction, believed to contain all the immunogenic activity of the intact organism(5), does not permit any conclusion as to the specific antigen responsible for the protection observed. The hemagglutinin on the other hand did not produce a good agglutinin response, nor did it protect against intracerebral challenge in active immunization experiments. It was somewhat surprising therefore to find that this fraction was capable of stimulating a booster response in mouse protective antibody against intranasal challenge. The further finding that the intracerebral protective antigen did not perform well in this study suggests that protection against intracerebral and intranasal manifestations of pertussis infection in mice depend on different mechanisms. Keogh and North(4) had originally postulated hemagglutinin as the protective antigen against intranasal challenge. Except for the present study, subsequent evidence(1,9), has not supported this view. Since in our experiments as well as those of Keogh and North, hemagglutinin was adsorbed on to stroma from crude extracts of the *H. pertussis* organism, it is possible that antigens other than hemagglutinin may have played a role in the mouse protective antibody response observed. The question arises where deductions can be made in regard to the relative value of these different antigenic agents in protecting against the disease. Admittedly extensive field trials are needed to answer the question definitely, but it would appear that the multiple antigens such as whole organisms or the soluble antigen have more to offer than single antigens for their greater ability to develop antibodies associated with protection against both intracerebral and intranasal challenge of mice. The value of antigens which fail to perform well in mouse protection tests, although they are good producers of other antibodies remains questionable.

Summary. A comparison has been made of the ability of 4 different pertussis antigens to produce a booster antibody response. Using increase of mouse protective antibody as measured by intranasal challenge as the criterion, excellent results were obtained with both soluble antigen and hemagglutinin. An explanation of the findings with the latter antigen is discussed.

We gratefully acknowledge the cooperation of Dr. Harold H. Berman and Dr. Martin Lazar of the Willowbrook State School in planning and carrying out this study. We also wish to thank Dr. Robert Ward and Dr. L. Emmett Holt, Jr., of this department, for their help and encouragement. Loretta Brown gave excellent technical assistance.

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Association of Echo Virus Type 6 with Aseptic Meningitis.* (22630)

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A number of viruses shown to be outside of the poliomyelitis, Coxsackie, and adenovirus groups, have been isolated from stool specimens of cases diagnosed as nonparalytic poliomyelitis, as well as from specimens obtained from normal children. Labelled *echo* (*enteric cytopathogenic human orphan*) *viruses,* they have been found to fall into at least 14 distinct antigenic types(1). The etiological relationships of the echo viruses to disease remain to be established. Evidence is presented here to show that one virus of the group, Type 6, seems capable of producing the aseptic meningitis syndrome. Suspicion of an etiological relationship was aroused when the virus was first recognized in 1954, for the first 3 strains isolated came from 3 cases of aseptic meningitis in Providence(2). We have found it only once among 212 enteric viruses isolated during a longitudinal study of *healthy* children(3,4).

Methods. Viruses were isolated and identified in monkey kidney cultures, and antibody determinations made, by methods already described(4). Specimens were collected as widely as possible in 1955 from patients in Connecticut hospitalized with the diagnosis of poliomyelitis, as a part of a study on the evaluation of poliomyelitis vaccine. They included a stool and serum sample obtained from each patient as soon after onset as possible and a second serum a few weeks later. Complement-fixing (CF) and neutralizing (Nt) antibodies against the polioviruses were determined on all sera collected. Nt antibody titrations were carried out using 4-fold dilutions of serum against 100 TCD_{50} of virus.

Results. Virus isolations. Fecal specimens were collected and tested from 58 paralytic and 137 nonparalytic patients. All but 4 of the specimens considered here were collected within 3 weeks of onset. In the nonvaccinated group of 144 cases, the ratio of paralytic to nonparalytic cases was 1 to 1.8, similar to that of previous years in this area, but markedly different from the ratio of 1 to 7.5 among the 51 vaccinated cases.

The results of the tests (Table I), may be summarized as follows: Poliovirus, chiefly type 1, was isolated from 39 of 52 nonvaccinated paralytic cases (75% positive) and from 3 of 6 vaccinated paralytic cases. In contrast, poliovirus was isolated in only 23 of 92 nonvaccinated nonparalytic cases (25% positive), and in only 5 of 45 vaccinated, nonparalytic cases (11% positive). There was no significant difference in the isolation of echo viruses or Coxsackie viruses in the vaccinated and nonvaccinated groups.

Of 45 viruses isolated from paralytic patients, 42 were poliovirus (39 type 1). Type 6 echo virus was isolated from a paralyzed patient, who probably had a mixed polio-echo infection. Even though poliovirus was not isolated, the Nt and CF antibody responses were those characteristic of poliomyelitis infection(5). Another paralyzed patient also was considered to have had a mixed infection, polio and Coxsackie A9. Although poliovirus was not isolated from this patient, it was isolated from his brother, and in addition, the patient gave Nt and CF antibody responses of poliomyelitis. A third case yielded only Coxsackie B4 virus, but as only a single acute phase specimen was obtained, the serum studies are incomplete.

Of 69 enteric viruses isolated from the nonparalytic cases, 28 (41%) were polioviruses

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† Epidemic Intelligence Service Officer, Communicable Disease Center, Atlanta, assigned to Yale Poliomyelitis Study Unit, New Haven.

TABLE I. Viral Isolations Obtained in Specimens Submitted from Cases of "Poliomyelitis" in Conn. during 1955.

	Nonvaccinated		Vaccinated		Total	
	Paralytic	Non-paralytic	Paralytic	Non-paralytic	Paralytic	Non-paralytic
Total cases studied (specimens collected within 3 wk of onset)[*]	52	92	6	45	58	137
No. yielding virus	42	45	3	24	45	69
Poliovirus Type 1	36	22	3	5	39	27
2	2	0	0	0	2	0
3	1	1	0	0	1	1
Echo virus Types 1, 2, 3, 4, 5	0	0	0	0	0	0
6	1[†]	9	0	12	1[†]	21
7, 8, 9, 10, 11, 12, 13, 14	0	0	0	0	0	0
Not typed	0	1	0	3	0	4
Coxsackie virus Type A9	1[‡]	1	0	0	1[‡]	1
B1	0	0	0	0	0	0
B2	0	5	0	1	0	6
B3	0	2	0	0	0	2
B4	1[§]	4	0	3	1[§]	7
B5	0	0	0	0	0	0

[*] Stool specimens from 10 paralytic cases collected between 22 and 115 days yielded 3 positives: one, a type 1 poliovirus isolated from a nonvaccinated case 50 days after onset; one, a type 2 poliovirus isolated from a nonvaccinated case 35 days after onset; and one, a type 1 poliovirus isolated from a vaccinated case 29 days after onset. Of 8 stools from nonparalytic cases collected between 22 and 45 days, one nonvaccinated case yielded type 1 poliovirus 22 days after onset. Of the late specimens, only these 4 are included in the results above.

[†] Probably mixed polio-echo type 6 infection. Although poliovirus was not isolated from the patient, CF and Nt antibody responses were characteristic of a polio-infection.

[‡] Probably mixed polio-Coxsackie A9 infection. Although poliovirus was not isolated from the patient, it was isolated from his brother. Furthermore, the patient gave a CF and Nt antibody response characteristic of polio-infection.

[§] Only Coxsackie B4 virus was isolated. An acute serum, the only one collected, failed to show polio-CF antibodies.

(27 type 1), 25 (36%) were echo viruses, and 16 (23%) were Coxsackie viruses. Of the Coxsackie viruses, 6 were B2, 2 were B3, 7 were B4, and only 1 was A9.[‡] Of the echo viruses, 21 were type 6, and 4 were unrelated to any of the 14 recognized types. Thus with the exception of poliovirus type 1, echo type 6 was encountered in this series more than any other virus.

When the nonparalytic cases are separated by age groups (Table II), the results may be summarized as follows: In the 5-10 age group, the only group vaccinated in 1955 in Connecticut, poliovirus was isolated from 5 of 45 vaccinated cases (11% positive), all type 1, and 6 of 22 nonvaccinated cases (27% positive). In the same age group, type 6 echo virus was isolated from 12 of 45 vaccinated cases (27% positive) and 6 of 22 nonvaccinated cases (27% positive). Coxsackie viruses were isolated from 4 of the vaccinated cases (9% positive) and 2 of the nonvaccinated cases (9% positive). Poliovirus was isolated from all of the age groups studied (with the exception of the 5 patients over 30 years). Similarly, Coxsackie viruses were found in all the age groups. However, the type 6 echo viruses were all isolated from children aged 5-13 years. The paralytic cases are not considered by age groups because most of the cases were positive and yielded poliovirus.

Serological findings. Multiple collections of serum were taken from the echo type 6 excretors, and Nt antibody titers were determined against the patient's own virus as well as against the D'Amori strain, the prototype

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[‡] In early spring 1956, however, when only 3 viruses were isolated in Conn. from the first 9 cases of aseptic meningitis seen, all 3 proved to be Coxsackie A9. This virus was isolated again in August from another patient with aseptic meningitis, Type A9 being obtained both from the stools and from the cerebrospinal fluid.

TABLE II. Viral Isolations from Nonparalytic Cases in Relation to Age.

Age groups	1–4	5–10 Non-vaccinated	5–10 Vaccinated	11–20	21–30	>30	Total
Cases studied	11	22	45	25	22	5	130
Poliovirus isolations	5	6	5	8	4	0	28
Echo-6 "	0	6*	12	3	0	0	21*
Coxsackie "	2	2	4	4	2	2	16

* Not included in this table are 3 additional cases from Providence, R.I., studied in 1954, which yielded echo-6 virus.

strain of echo type 6 virus. As similar homologous and homotypic titers were found, hereafter only the titers against the prototype strain are considered. The results of the tests, as shown in Table III, may be summarized as follows: Echo-6 excretors produced homotypic antibodies more slowly, and at lower titer than the poliovirus excretors produced homotypic polio-antibodies. Polio-antibody titers were essentially unaffected in excretors of echo-6 virus.

Specific examples giving vaccination status, poliovirus Nt and CF, and echo-6 Nt antibody titers are listed in Table IV. All of these cases were excreting echo-6, the stool being collected at the time the first serum was drawn. The first two patients listed were not vaccinated. Each demonstrated poliovirus Nt antibodies to a single poliovirus type, without a change in titer being noted during the course of the disease. As the CF tests were negative, the Nt antibodies are assumed to represent a past subclinical polio-infection, for no history of poliomyelitis was obtained. Other patients failed to have any detectable antibodies against any of the poliovirus types. Two patients listed had been vaccinated, each having received 2 doses of formalinized vaccine, about 2 months prior to infection with the echo virus, which probably accounts for their elevated levels of poliomyelitis Nt antibodies. Again the polio-antibodies remained constant during the course of the illness. In contrast all 4 patients demonstrated a rise in antibodies to echo-6 virus, as did all of the echo-6 excretors from whom adequately spaced serum specimens were collected.

TABLE III. Neutralizing Antibody Titers* in Aseptic Meningitis Patients (Age 5-13 Years) Infected with Echo Virus Type 6 or Poliovirus Type 1.

	Acute, 1-7 days†	Convalescent, over 7 days†
Echo-6 donors		
Nonvaccinated sera tested	9 pairs	
Echo-6 serum titers	<10	100
Polio-1 " "	50	50
Vaccinated sera tested	11 pairs	
Echo-6 serum titers	<10	50
Polio-1 " "	256	256
Polio-1 donors		
Nonvaccinated sera tested	4 pairs	
Polio-1 serum titers	150	200
Vaccinated sera tested	3 pairs	
Polio-1 serum titers	100	200

* Geometric mean.

† Interval in days between onset of illness and collection of serum. Only paired sera in which the acute specimen was drawn during first week are included.

Signs and symptoms. Clinical data were obtained from the patients studied. Only the results of the study of aseptic meningitis cases associated with echo-6 virus, the Coxsackie group B viruses, and poliovirus-1 are shown in Table V, where the cell counts of the cerebrospinal fluids are also noted. It may be seen that the symptomatology among the 3 groups was not significantly different. Fever, malaise, headache and stiff neck were most commonly encountered. Stiff back, back pains, muscle pain, vomiting and sore throat were occasionally encountered, and abdominal pains and constipation were infrequently found. An increased number of cells in the spinal fluid was noted in all excretors of echo-6 virus in which a lumbar puncture was performed. The number of cells ranged from 12 to over 300 with about 75 cells per c. mm. being the average count. Eight of 11 Coxsackie excretors showed increased cells in the spinal fluid. Twenty of 23 poliovirus-1 excretors showed increased cells in the spinal

TABLE IV. Titers of Polio-Types I, II and III and Echo-6 Antibodies in Paired Sera of Aseptic Meningitis Patients Excreting Echo-6 Virus.

		Poliovirus antibody titer						Echo-6 neutralizing antibody titer
		Neutralization			Complement fixation			
Age	Days after onset	I	II	III	I	II	III	
6 (nonvaccinated)	3	<4	<4	64	0	0	0	<10
	45	<4	<4	64	0	0	0	100
6 "	1	256	<4	<4	0	0	0	<10
	31	256	<4	<4	0	0	0	100
8 (vaccinated)	5	256	1024	1024	0	0	0	<10
	12	256	1024	1024	0	0	0	50
6 "	4	64	1024	16	0	4	0	10
	18	64	1024	16	0	4	0	100

fluid. Here again, by use of the cell count, no significant differences were present between the 3 groups.

Discussion. Even though it has long been known that aseptic meningitis may be caused by a variety of agents(6,7), a large proportion of cases are still of unknown etiology. With the increasing use of tissue culture technics, a number of new agents have been isolated from patients manifesting this syndrome. This might well be merely a reflection of the type of case studied. Before a newly recognized agent can be said to be the etiological agent of a disease, it must be shown to have a high correlation with the occurrence of the illness. The situation is not simple in the case of a syndrome having a diverse etiology and a virus which does not produce disease in laboratory animals. Nevertheless, the present report of echo virus type 6 as the only agent associated with 24 cases of the disease lends strong support to the view that this virus is one of the etiological agents of aseptic meningitis. Infections produced with the virus in this series of cases were of the classical type, in that antibody rises were readily demonstrated in paired sera. At the recent Conference on Viruses in Search of Disease, 3 different laboratories(8,9,10) also commented on finding a high degree of association of echo-6 virus with aseptic meningitis cases, including the isolation of the agent from the cerebrospinal fluid in some of the patients studied(9).

TABLE V. Clinical Features of Aseptic Meningitis Patients Associated with Echo-6, Coxsackie B and Poliovirus Excretion.

Signs and symptoms	Echo-6	Coxsackie B	Poliovirus
Fever and malaise	21/21	11/13	19/20
Headache	17/21	11/13	17/20
Stiff neck	18/21	12/13	17/20
" back	8/21	3/13	10/20
Back pain	4/21	3/13	8/20
Muscle "	4/21	3/13	7/20
Vomiting	15/21	5/13	10/20
Abdominal pain	3/21	0/13	0/20
Sore throat	5/21	4/13	10/20
Diarrhea	0/21	0/13	0/20
Constipation	0/21	1/13	3/20
CSF pleocytosis (LP performed 1-10 days after onset with the majority being done on 2nd day)			
Less than 10 cells	0/18	3/11	3/23
10- 24 cells	1/18	0/11	1/23
25- 99 "	9/18	4/11	8/23
100-300 "	5/18	1/11	8/23
Over 300 "	3/18	3/11	3/23
More than 10 cells	18/18	8/11	20/23

Summary. In a study of vaccinated and non-vaccinated "poliomyelitis" patients in Connecticut during the summer and fall of 1955, 78% of the paralytic, but only 50% of the nonparalytic, cases yielded viruses cytopathogenic for monkey kidney cultures. Of the 45 agents isolated from paralytic patients, 93% were polioviruses, chiefly type 1. Two patients had mixed infections: echo-6 and polio-1, and Coxsackie A9 and polio-1. Of the 69 enteric agents isolated from the nonparalytic cases, 41% were polioviruses (type 1, with a single exception), 23% were Coxsackie viruses (B2, B3, B4, A9), and 36% were echo viruses. Of the 25 echo viruses, 21 belonged to echo-6 and 4 were unrelated to any of the 14 recognized echo types.

In addition, echo-6 was recovered from 3 cases of aseptic meningitis in Rhode Island during the summer of 1954. Together with evidence being obtained in other laboratories, these data strongly support the view that echo virus type 6 is one of the etiological agents of the aseptic meningitis syndrome.

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Epinephrine-Chlortetracycline Induced Arrhythmia in the Isolated Rat Heart. (22631)

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(Introduced by C. H. Hine.)

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The effect of antibiotics on the host has received considerable attention. Their possible effect on cardiac action has been emphasized. One of the authors, with another worker(2) found that epinephrine-chlortetracycline induced arrhythmia can be demonstrated in isolated frog and turtle hearts. This led to the experiments reported here on the effect of epinephrine-chlortetracycline on the isolated mammalian heart.*

Methods. A white rat was killed by a blow at the base of the skull. The heart was excised and the coronary arteries were perfused through a modified Straub cannula in the aorta. The heart was allowed a 10-15 minute stabilization period and its contractions were recorded kymographically. After an additional 10 minutes, the drug was administered by injection into the perfusate and in approximately 20-25 minutes following this a challenge dose of epinephrine (1 μg) was added to the perfusate.

The temperature for each experiment was kept to within ± 0.5°C and ranged between 34-37°C in different experiments. The perfusate was well-oxygenated Lockes' solution; a glucose concentration of 0.2% strength rather than the lower limit of 0.1% aided in reducing irritability and in stabilizing the heart (Fig. 1). The elapsed time per experiment averaged 1.9 hours although a normal record without irregularity of beat for up to 3.4 hours was made.

Results. An isolated rat heart treated with 100 mcg of chlortetracycline-HCl† followed by 1 μg of epinephrine developed both inotropic and chronotropic irregularities (Fig. 2) in which the normal beat was interrupted for from 5-30 minutes, usually 15-20 minutes, by one or more of several different kinds of arrhythmia as follows: 1. Cyclic variation in amplitude; 2. Periods of increased amplitude followed by 2-20 dropped beats or by contractions of smaller amplitude; 3. Series (2-5) of beats irregular in rate and amplitude; 4. Complete cessation of beat (Fig. 5).

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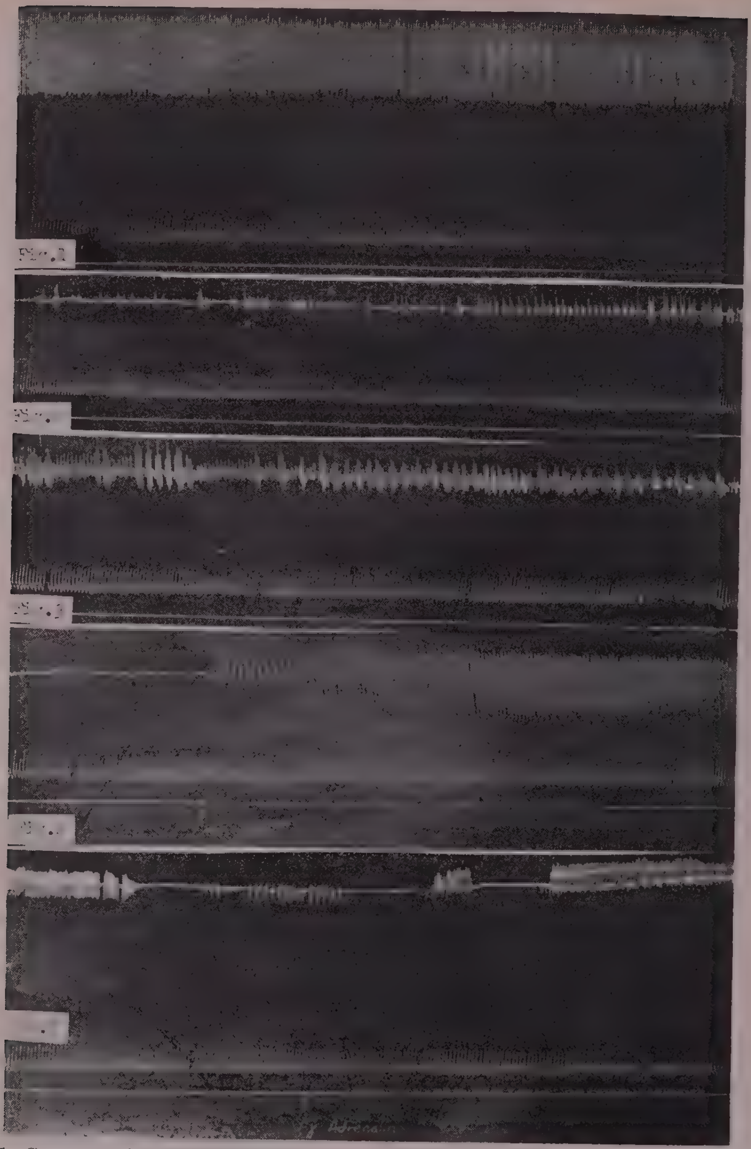

FIG. 1. Segment of normal kymographic tracing of beat of isolated rat heart 0.9 hr following theoretical drug addition. The entire tracing resembled this segment.

FIG. 2. Effect of 100 μg of chlortetracycline-HCl on beat of isolated rat heart 1.3 hr following addition of drug.

FIG. 3. Effect of 20 μg of tetrasodium versenate on beat of isolated rat heart 1.0 hr following addition of drug.

FIG. 4. Alleviation of arrhythmia in isolated rat heart by 40 μg of Mn^{++} (as $MnCl_2, 4H_2O$) 1.2 hr following addition of 100 μg of chlortetracycline-HCl.

FIG. 5. Cessation of beat of isolated rat heart due to 500 μg of chlortetracycline-HCl.

(Timer markings in seconds in all tracings.)

Twenty μg of a known chelating agent, tetrasodium versenate, were added to the perfusate in place of chlortetracycline-HCl in 4 experiments. Cardiac irregularities developed which were almost identical with irregularities induced by chlortetracycline-HCl and which appeared in approximately the same time intervals (Fig. 3). In 3 experiments, the addition of 40 μg Mn^{++} as manganous chloride to the perfusate following onset of arrhythmia that had been induced by chlortetracycline alleviated the irregularities temporarily with resumption of a normal beat (Fig. 4).

Discussion. To account for the effect of chlortetracycline-HCl on the isolated frog heart, Yang and Harvey(5) proposed alteration of acetylcholine regulation of cardiac action as a possible explanation. If alteration of acetylcholine control resulted in accumulation of acetylcholine, the inhibitory effect of this mediator on heart rate would occur and inotropic and chronotropic irregularities would appear. Alteration of acetylcholine regulation of cardiac action might result from chelation of the co-factor of cardiac cholinesterase by chlortetracycline. Thus, cholinesterase would be unable to destroy acetylcholine which would accumulate and cause cardiac irregularity. Weidenheimer, Reed and Upham(4) demonstrated the ability of chlortetracycline to form complexes with 11 different metallic ions. Mn^{++} was not included in this list. However, Albert(1) showed that chlortetracycline will chelate Mn^{++} and Saz and Slie(3) found that Mn^{++} will reverse the inhibition of nitro reductase by chlortetracycline.

Summary. The effect of chlortetracycline-HCl on the isolated rat heart is practically identical with the effect of a known chelating agent, tetrasodium versenate. This effect can be reversed temporarily by Mn^{++}. Thus, chlortetracycline seems to cause irregularities in the isolated rat heart by chelation of the co-factor of cholinesterase which inactivates this enzyme and permits acetylcholine to accumulate and to exert its inhibitory effect on the heart. The co-factor of cardiac cholinesterase may be Mn^{++}.

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Regulation of Heat Production in Cold-Adapted Rats.* (22632)

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Recent studies(1,2) of adaptive changes induced in small mammals by prolonged exposure to cold have indicated that there is a reduction of muscular activity after adaptation. This demonstration along with the observation that heat production in the cold is increased(3,4) suggests that a regulatory mechanism not dependent on excitation of skeletal muscle is effective after adaptation. Such control of metabolic heat production was postulated by Rubner(5) to account for increased heat production not accompanied by increased muscular activity.

Regulation not due to muscular activity has been postulated to be mediated through release of epinephrine from adrenal medulla (6,7). The increase in such thermogenesis on cold adaptation may be due either to greater release of epinephrine or to greater sensitivity of adapted animals to the hormone.

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To test capacity of non-shivering means of regulating heat production after adaptation to cold, the metabolic response to cold of cold-adapted rats whose muscular activity was blocked by curare, was compared to that of similar nonadapted rats. To evaluate the role of the adrenal medulla in this response a number of adrenodemedullated rats were subjected to similar tests.

Methods. Male Sprague-Dawley rats were kept in individual metal cages, fed Purina ground chow, and given water without restriction. Eight rats were placed in a cold room at 5°C ± 1° for approximately 60 days. Eight control animals were kept at 28°C ± 2°. The lights in the cold room were regulated to approximate the normal day-night conditions of the room in which the non-adapted animals lived. Body weights ranged from 170-220 g at the time the rats were placed in the cold and 300-400 g at the end of the experimental period.

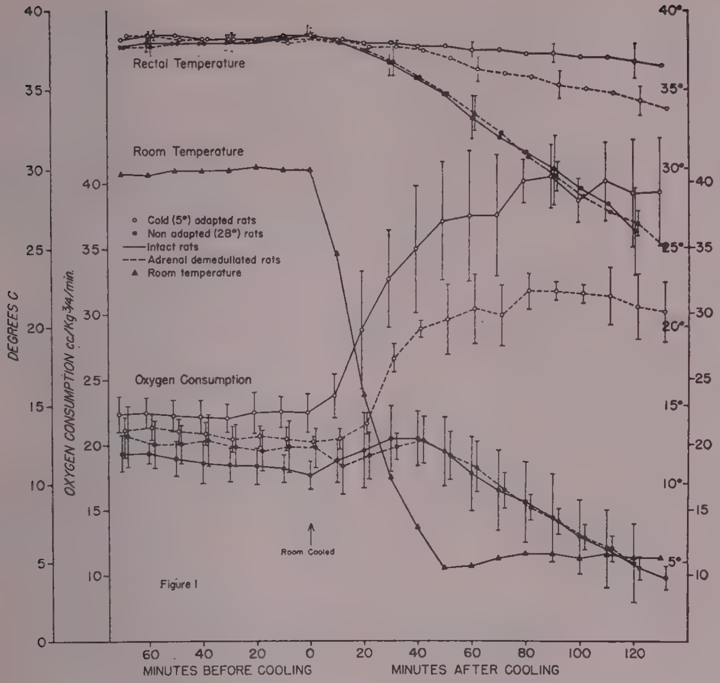
Thirty-five days after the animals had been placed in the cold, 4 were removed and along with 4 from the warm room were adrenodemedullated. Animals from the cold room were returned 8 hours after demedullation. Both groups were provided with 1% NaCl as drinking water for 10 days. At autopsy, the demedullated adrenals were removed and prepared for histological study.

At the end of the exposure period the metabolic response of each animal while curarized was tested separately. For this, the animal was prepared in a test room at 30°C. With the animal lightly etherized and supine on a board, the trachea was cannulated and the ventilatory pump connected. A dose of 360 μg of d-tubocurarine was then injected into the external jugular vein. Oxygen consumption was determined using an open circuit in which the expired air was passed through a Beckman Oxygen Analyzer and in which flow rate was determined by timing a sample of the expired air collected in a small spirometer. Output of the pump was regulated to maintain an expired oxygen tension of 120-130 mm Hg. Rectal, foot, and ambient air temperatures were measured with copper-constantan thermocouples recording on a Brown potentiometer. Electromyograms (EMG) and electrocardiograms (EKG) were recorded from needle electrodes inserted into muscles of the fore- and hindlimbs. When activity appeared in the EMG, an additional 180 μg tubocurarine were injected. For initial 90 minutes of the test (70 of which are indicated in Fig. 1) the room temperature was held at 30°C. It was then lowered to 5°C over a period of 30 to 40 minutes, and held at 5°C for an additional 100 minutes or until rectal temperature of the animal had dropped to 25°C. Throughout the test period, readings were made at 10-minute intervals. After the test each animal was rewarmed by radiant heat to its initial body temperature, and its oxygen consumption compared with that during the initial period to check the condition of the preparation.

Results. All rats adapted to 5°C were able to increase their heat production when exposed to cold even though muscular activity was blocked with curare (Fig. 1). In contrast, non-adapted animals were unable to increase heat production sufficiently to prevent a marked fall in rectal temperature.

The increase in heat production by the intact cold-adapted animals was sufficient to maintain rectal temperature at or near the initial level. Demedullated cold-adapted rats also increased their heat production but significantly less than that of the cold-adapted animals. Their rectal temperature fell. The response of the demedullated warm-adapted animals did not differ from that of the intact non-adapted. Histologically, the demedullated adrenals showed well-developed cortical tissue with the medulla replaced by scar tissue.

When the room temperature was 30°C, foot temperatures of the cold-adapted animals approximated the rectal temperatures, evidence of marked peripheral vasodilation. This is shown in Fig. 2 as a large difference between foot and ambient air temperatures. The foot temperatures of the nonadapted animals approximated ambient air temperature, evidence of peripheral vasoconstriction. As the room cooled, the temperature gradient from foot to air increased transiently, owing to different



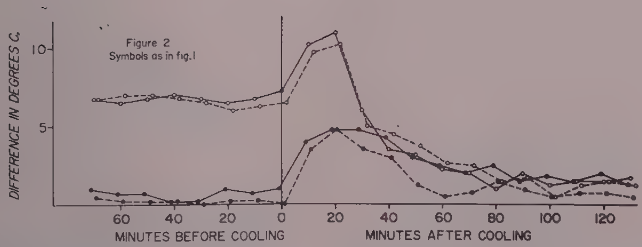


FIG. 1. Metabolic response of curarized rats to lowered ambient temperature. Mean oxygen consumption and rectal temperature of each group along with ambient temperature is plotted on ordinate; minutes before and after cooling on abscissa. Stand. dev. are indicated by vertical lines.

FIG. 2. Vasomotor responses of curarized rats to lowered ambient temperature. Temperature gradient between the feet of preparations and ambient temperature is plotted on ordinate; minutes before and after cooling on abscissa.

cooling rate of air and tissue and to delay in the onset of vasoconstriction. After cooling was completed, all animals showed marked vasoconstriction.

Discussion. The response observed indicates that the capacity of the non-shivering control of heat production is greater after adaptation to cold. This increase supports the suggestion(8) that this regulation of body heat is increased with adaptation to cold. Such a mechanism of augmenting metabolic heat production would have a sparing action on muscular activity and therefore would account for the reduction in the integrated electrical activity of skeletal muscle of rats(1,2) or the change in the electromyographic patterns of rabbits(9) after adaptation to cold. It would also account for the quieter and more comfortable appearance of adapted animals in the cold(3) and for the ability of the adapted animals to maintain the high level of heat production enabling them to survive severe cold stress(10,11,12). The response of the cold-adapted adrenodemedullated rats indicates that the non-shivering regulation in the cold-adapted rat is mediated only in part by release of epinephrine from the adrenal medulla. If the balance of the response is produced by epinephrine then it must be due to epinephrine released by extra-adrenal medullary tissue or sympathetic endings for there was no evidence of regeneration of adrenal medullary tissue.†‡ In adrenodemedullated rats kept at room temperatures Vogt (13) and Bloom and Russel(14) found little or no increase in circulating epinephrine from conditions which caused its release in normal animals. The response of the cold-adapted demedullated rats to cold suggests that following adaptation more extra-adrenal epinephrine is produced either as a result of an increase in the amount secreted or a change in the ratio of norepinephrine to epinephrine produced. The response also may be to some extent a product of the increased sensitivity of cold-adapted rats to epinephrine. One plausible explanation of our results is suggested by the work of Ring(15) and of Swanson(16). The greater effectiveness of the non-shivering regulation after adaptation to cold could result from an increased sensitivity to the endogenous epinephrine which has been shown(6,7,17) to be released by animals on exposure to cold. The potentiation of the epinephrine response may be related to the increased thyroxine requirement of cold-adapted animals(18,19). The increase may be analogous to that seen in animals made hyperthyroid by administration of thyroxine (16,17).

Summary. (1). The capacity of cold-adapted and non-adapted rats to increase heat production by non-shivering regulatory means was tested. In both groups, muscular activity was blocked by curare. Only cold-adapted animals could increase heat production sufficiently to maintain body temperature as the test room was cooled. Adrenodemedullation reduced somewhat the ability of the cold-adapted rats to increase heat production, but made little change in the response of non-adapted animals. It is suggested that this regulation results from the release of adrenal and extra-adrenal epinephrine and that the increased heat production by cold-adapted animals results from an increased epinephrine sensitivity. (2). To our knowledge this is the first demonstration of non-shivering heat regulation of this magnitude. The response is augmented by adaptation to cold.

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† Two acute spinal (C3) animals, 1 adapted and 1 nonadapted, cooled immediately when room temperature was lowered. Although the muscle in the heads of these animals showed shivering responses, oxygen consumption did not increase.

‡ Two acutely adrenalectomized cold-adapted rats also showed a marked response, suggesting that response of the demedullated animals did not result from compensatory changes.

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Effect of Fluorides and Other Solutions on Solubility of Powdered Enamel in Acid. (22633)

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Considerable interest has been shown recently in the sarcosinates and in dehydroacetate as possible inhibitory agents of human dental caries. It has been suggested that mechanism of action in this regard is the inhibition of acid formation in the mouth through interference with enzyme activity(1). However, a recent publication(2) suggests that the sarcosinates are as effective as fluorides in decreasing the solubility of powdered enamel in acid and, thus, may be of importance in prevention of dental caries by this mechanism also. Since stannous fluoride has been shown to be considerably more effective than sodium fluoride in reducing enamel solubility(3), experimental dental caries in rats(4) and hamsters(5), and human dental caries when applied topically(6) or in a dentifrice(7), it was of interest to compare sodium N-palmitoyl sarcosinate with stannous fluoride. Similarly, sodium oxalate(8) has been suggested as possessing "fluoride-like" protection action on enamel, and it was of interest to compare this compound with stannous fluoride and sodium N-palmitoyl sarcosinate.

Methods. The procedure used for measuring the effectiveness of the 5 compounds has been described(3,9). In this study, a 5.0-ml portion of an aqueous solution of the compound to be studied was added to a 100-mg portion of powdered enamel. In all instances the solutions were used as prepared without adjustment of the pH. After the mixture was shaken for 20 minutes at 25°C it was promptly centrifuged. The clear supernatant was decanted and 1.0 ml of redistilled fluoride-free water was used to wash the enamel. The supernatant and the wash solution were combined and suitable aliquots were analyzed for phosphorus(10). This served as a measure of the amount of decalcification occurring during the treatment period with any of the proposed protecting solutions. Following this, 10.0 ml of 0.2 N acetic acid (pH 4.0) was added to the powdered enamel, and the mixture was shaken for 20 minutes as before. Following prompt centrifugation the supernatant solution was decanted, the enamel was washed. and the combined solutions were analyzed for phosphorus. Controls were run in the same manner except that distilled water was used in place of the treatment solutions.

The amounts of phosphate dissolved by the treatment solution (fluoride, sarcosinate, dehydroacetate, or oxalate) and by the decalcification solution (acetic acid) were combined to give a figure representing the total amount of tooth substance lost. The effectiveness of each treatment solution was then calculated by comparing this value with the corresponding control value (for untreated enamel), and expressing the result as percentage reduction in solubility.

Results. The data obtained are presented in Table I. The stannous fluoride was by far the most effective compound tested; a 1% solution reduced enamel solubility by 86%, and a 0.1% solution was 85% effective. Under similar conditions, sodium fluoride reduced enamel solubility by 44 and 36%, respectively. The sodium N-palmitoyl sarcosinate was only moderately active, a 1.0% solution reducing the solubility of the powdered enamel by only 20% and a 0.1% solution by only 4%. Volker *et al.*(2) also evalu-

TABLE I. Effects of Stannous Fluoride, Sodium Fluoride, Sodium N-Palmitoyl Sarcosinate, Sodium Dehydroacetate, and Sodium Oxalate on Solubility of Powdered Tooth Enamel in Acid.

Compound	Concentration (%)	pH	No. of samples used	P lost during protection	P lost during acid treatment	Reduction in enamel solubility (%)
				(mg P/100 mg enamel)		
Stannous fluoride	1.0	3.5	10	.09	.38	86.6 ± 1.9*
	.1	3.7	12	.01	.50	85.4 ± 1.6
Sodium "	1.0	7.2	10	.14	1.80	44.3 ± 4.1
	.1	6.7	14	.40	1.84	36.5 ± 4.0
" N-palmitoyl sarcosinate†	1.0	8.5	12	.55	2.25	20.0 ± 3.5
	.1	8.2	14	.16	3.20	4.0 ± .9
" dehydroacetate	1.0	3.8	8	.36	2.96	5.0 ± 1.1
	.1	4.1	8	.30	3.13	2.0 ± .2
" oxalate	1.0	6.3	10	.10	3.36	1.0 ± .02
	.1	6.9	10	.37	3.20	—
Control (distilled water)		5.5	24	.40	3.10	—

* Stand. dev. † The purity of this compound was 95.3%.

ated a 0.01% solution, but, since a 0.1% solution was so ineffective in these studies, the lower concentration was not investigated. Sodium dehydroacetate and sodium oxalate were not effective to any appreciable degree in reducing the solubility of powdered dental enamel.

Discussion. These data demonstrate that, under the conditions of this study, the 2 fluoride salts are markedly superior to sodium N-palmitoyl sarcosinate and dehydroacetate in their ability to decrease the solubility of powdered enamel, and that stannous fluoride is considerably more effective than sodium fluoride. Sodium oxalate is without effect. The results with the sarcosinate are in contrast to those of Volker *et al.*(2), who found the effect of this compound to be equal to that of sodium fluoride. In the present study, the sarcosinate was essentially ineffective even at 10 times the concentration tested by Volker *et al.* This discrepancy is particularly difficult to understand since results obtained for sodium fluoride were quite similar in the two investigations. Since most studies of this nature serve only to screen potential anticariogenic substances, one should accept all such results as presenting only suggestive evidence as to how they might act in reducing human dental caries. The final criterion for such an evaluation must be the demonstration of effectiveness by dental caries studies. However, laboratory studies are of considerable value in predicting potentially useful compounds. It is interesting, however, to note that studies have already indicated the superiority of stannous fluoride over sodium fluoride when applied topically(6) or when incorporated in a dentifrice(7). The suggestion of usefulness for the sarcosinate on the basis of enamel solubility reduction is not in keeping with the proposed mechanism for reduction of caries by an antienzyme action. Indeed, the present data cannot be interpreted as suggesting a fluoride-like action for the sarcosinate, dehydroacetate, or sodium oxalate.

Summary. The solubility of powdered human tooth enamel was measured after treatment with stannous fluoride, sodium fluoride, sodium N-palmitoyl sarcosinate, sodium dehydroacetate, or sodium oxalate by a method which measures the amount of phosphorus dissolved from the treated enamel by acetic acid. The data indicate that stannous fluoride is markedly superior to sodium fluoride and that the sarcosinate is of only questionable value in its ability to reduce powdered enamel solubility. Sodium dehydroacetate and sodium oxalate were without effect in decreasing the solubility of powdered enamel.

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Chlorpromazine Counteraction of Trauma Induced Death.* (22634)

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Chlorpromazine(1) has been reported to have a pronounced protective action against death induced in dogs by severe hemorrhage (2,3) and in rats by tumbling trauma(3). This paper deals with the counteraction by chlorpromazine of hemoconcentration and death induced in mice by tourniquet trauma, and by heat trauma. An abstract of part of this work has already appeared(4). Findings similar to certain of those reported here have been published recently by Millican and Stohlman(5).

Methods. Mice used were Carworth Farms males, 2-5 months of age and 20-30 g weight. In any one experiment mice were sorted into such groups that the average body weights for all the groups were nearly the same. Traumatization procedures were those of Rosenthal(6,7). In the tourniquet trauma experiments the hind leg tourniquets were left in place 2 or 3 hours; in the heat trauma experiments etherized mice were submerged to the neck in water at 60°C for 12 seconds. All injections were made intraperitoneally. Chlorpromazine was dissolved in distilled water at such a concentration that the desired dose was secured by injection of .01 ml per g, or in .9% NaCl at such a concentration that the desired dose was secured by injection of .05 ml per g. Since initiation of shock presumably begins at the time of removal of hind leg tourniquets, or at the time of immersion in very hot water, this time has been designated *zero hour* (Tables). Prior times have been assigned minus (—) values, later times plus (+) values. For the present purposes shock in mice is defined as the condition after severe traumatization wherein mice are prostrated, relatively indifferent to stimuli, cold to the touch, and likely to die within 48 hours after traumatization if untreated. *Hematocrit studies*: Hematocrit values were obtained using heparinized fine capillary tubes and an International "Hemacrit" centrifuge. A control tail blood sample was secured for each mouse. In all except the earliest experiments those mice exhibiting tail blood hematocrit values below 40 were replaced. The second (test) blood sample was taken from the heart, since insufficient blood could be obtained from the tail after traumatization. Hemodilution was indicated by a heart blood hematocrit value appreciably smaller than the previously obtained tail blood hematocrit value, hemoconcentration by the reverse. For any one group, the standard error of the average difference between tail and heart blood hematocrit values was calculated using this difference for each mouse as the unit. The probability value, p, was secured from a (t,

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TABLE I. Counteraction by Chlorpromazine of Tourniquet Trauma Induced Hemoconcentration.

Procedure timing, in hr, before (−) or after (+) removal of hind leg tourniquets			Avg differences between tail and heart blood hematocrit values (DHV's)*			
			Dist. water inj.		Drug inj. (8 μg/g)	
Ligatures on	Drug or DW inj.	Mouse sacrificed	No. of mice†	DHV's*	No. of mice†	DHV's*
−2	−2.5	+ 2	4/ 4	+ 6.9	4/4	−17.5
		+ 6	3/ 4	+ 5.5	4/4	− 4.5
		+10	4/ 4	+ 3.3	4/4	+ 4.8
−2	−1	+ 2	8/ 9	+ 9.8	6/6	+ 2.3
		+ 4	7/ 9	+13.9	7/8	+ 5.0
		+ 6	10/12	+16.9 ± S.E. 1.87‡	8/8	+ 4.8 ± S.E. 1.56‡
		+10	3/ 4	+15.2	3/4	+ 7.0
−3	−1	+ 2	4/ 4	+20.0	4/4	+ 5.1
		+ 4	3/ 4	+21.8	4/4	+ 6.9
		+ 6	3/ 4	+15.0	4/4	+ 7.4
		+10	6/ 9	+14.7	6/9	+12.4

* DHV marked plus (+) if heart blood hematocrit exceeded tail blood hematocrit, and minus (−) if vice versa.

† Numerator, No. of mice surviving to time of sacrifice; denominator, No. of mice alive at beginning of experiment.

‡ For mice of this experiment, sacrificed 6 hr after removal of hind leg tourniquets, the probability that chlorpromazine, administered as indicated, did not counteract tourniquet trauma induced hemoconcentration is estimated as less than .001.

n) table(8), t being calculated in the usual manner. *Survival studies*: Each experiment was set up to permit calculation of Chi Square. The value p was then secured from a one degree of freedom Chi Square table(8).

Results. 1. *Hematocrit studies*: For 25 control mice an average tail blood hematocrit value of 46.22 ± S.E. 0.81% and an average heart blood hematocrit value of 44.12 ± S.E. 0.91% was obtained, p value for difference over .05. No effect of varying time between tail and heart blood samples, from 0 to 48 hours, was noted. *Chlorpromazine on hematocrit values of normal (non-traumatized) mice*: Mice were sacrificed at intervals ranging from 3 to 11 (usually 6) hours after administration of water or drug. No differential effect of time was noted. Average hematocrit values obtained for 35 mice injected with water were: (a) tail blood, 45.9; (b) heart blood minus tail blood, —3.86 ± S.E. .44. Average hematocrit values obtained for 34 mice injected with chlorpromazine, 8 μg per g, were: (a) tail blood, 46.4; heart blood minus tail blood, —8.16 ± S.E. .62. The probability that the moderate hemodilution associated with the use of the drug was due to chance is estimated to be less than .001. *Chlorpromazine on tourniquet trauma induced hemoconcentration*: When administered either prior to application of hind leg tourniquets or after their application but prior to their removal, chlorpromazine at a dose level of 8 μg per g was effective in some degree in counteracting tourniquet trauma induced hemoconcentration (Table I). During these experiments a few limp mice were encountered which failed to show a beating heart when the chest was opened. Data obtained for these moribund mice were not used in connection with Table I. However, it is interesting to note that appreciable hemoconcentration was encountered only for heart blood samples of those moribund mice which had been injected with water rather than the drug, prior to removal of the hind leg tourniquets. Drug counteraction of trauma induced hemoconcentration was not entirely equivalent to prevention of trauma induced death. In still other experiments it was found that if the drug was administered after *removal* of the hind leg tourniquets, no appreciable counteraction of the trauma induced hemoconcentration occurred. *Chlorpromazine on heat*

TABLE II. Effect of Chlorpromazine Alone on Survival of Mice Subjected to Tourniquet Trauma.

Exp.	Dose of drug (µg/g)	Timing of procedures, in hr, before (−) removal of hind leg ligatures		Survivals after removal of hind leg ligatures				p from chi square (48 hr)*
		Ligatures on	Drug or DW inj.	Up to 24 hr		Up to 48 hr		
				No. of mice†	% of mice	No. of mice†	% of mice	
A	None	−2	−2½	14/41	34	13/41	32	
	8	−2	−2½	44/52	85	43/52	83	<.001
B	None	−2½	−2½‡	22/43	51	18/43	42	
	8	−2½	−2½‡	34/44	77	34/44	77	<.001
C	None	−2	−2 ‡	4/13	31	3/13	23	
		"	−1½	1/12	8	1/12	8	
		"	−1	5/12	42	4/12	33	
		Totals for controls:		10/37	27	8/37	22	
	8	−2	−2 ‡	23/25	92	23/25	92	<.001*
		"	−1½	19/25	76	19/25	76	<.001*
		"	−1	17/25	68	16/25	64	.001*
D	None	−2	−1	5/15	33	2/15	13	
	8	−2	−1	10/25	40	2/25	8	.58 Not significant

* In Exp. C, each treated group was compared with the *entire* control group.

† Numerator, No. of survivors; denominator, No. of mice alive at start of experiment.

‡ Injections made immediately after application of hind leg ligatures.

trauma induced hemoconcentration: Two experiments were performed in which mice were injected with water or drug, 8 µg per g, 1 hour *before*, and heart blood samples secured 2 hours after heat traumatization. Values for the factor "increase in hematocrit occurring in water injected mice, *minus* increase in hematocrit occurring in drug injected mice" were as follows: Exp. 1, + 8.7 ± S.E. 2.74; Exp. 2, + 5.19 ± S.E. 1.62. In both experiments, t (factor/S.E. of factor) was 3.2 and the corresponding p less than .02. It is concluded that *pre*-administration of chlorpromazine resulted in significant counteraction of heat trauma induced hemoconcentration. However, significant counteraction of heat trauma induced hemoconcentration was not secured in two experiments in which the drug was administered at a dose level of 8 µg per g immediately *after* heat traumatization.

Survival studies: A. *Chlorpromazine alone on tourniquet trauma induced mortality*: The data of Table II indicate that maximum protection was secured by administering the drug before application of hind leg ligatures, as in in Exp. A, or immediately thereafter, as in Exp. B and C, and that protection became progressively weaker as the time of drug administration was moved away from time of application of hind leg ligatures toward the time of their removal.

B. *Chlorpromazine supplemented with isotonic saline on tourniquet trauma induced mortality*: The lessening of tourniquet trauma induced hemoconcentration which was secured by use of chlorpromazine administered some time after placement of hind leg tourniquets, but before their removal (Table I) suggested that the drug may delay onset of irreversible shock, even when administered such a short time before removal of hind leg tourniquets that it might be ineffective, by itself, in promoting indefinite survival. If so, administration of large volumes of isotonic saline some time after shock had been precipitated by removal of the hind leg tourniquets should result in a survival difference between control and chlorpromazine injected mice in favor of the latter, even when the drug alone was ineffective in promoting survival. A number of experiments were performed in which each mouse was injected with either water or the drug, 8 µg per g either 15 or 60 minutes before removal of the hind leg tourniquets, and with .9% NaCl, in the amount of

5% of the body weight, 2 hours after removal of the tourniquets. Of 69 mice injected first with water and later with saline, 22 survived to 24 hours, and 13 to 48 hours after removal of tourniquets. Of 68 mice injected first with the drug and later with saline, 42 survived to 24 hours and 32 to 48 hours after removal of tourniquets. The probability, p, that in these experiments the drug plus saline did not enhance likelihood of survival to 48 hours over that obtainable with saline alone is estimated to be less than .001.

Chlorpromazine plus saline failed to enhance survival over that obtainable with saline alone, when both drug and saline were administered *after removal* of hind leg tourniquets. For example, in an experiment in which injections were made 2 hours after removal of tourniquets, survivals to 48 hours were as follows: 16 of 30 mice injected with saline only, 5% of body weight; but only 4 of 30 mice injected with saline plus chlorpromazine, 8 μg per g.

C. *Chlorpromazine on heat trauma induced mortality.* Of 50 mice injected with water one hour before traumatization, 21 survived to 24 hours, and 18 to 48 hours after traumatization. Of 40 mice injected with the drug, 8 μg per g, one hour before traumatization, 40 survived to 24 hours and 35 to 48 hours. The probability that the drug, administered one hour *before* traumatization, did not increase likelihood of survival to 48 hours is estimated to be less than .001.

Of 75 mice injected with distilled water or isotonic saline immediately *after* heat traumatization, 29 survived to 24 hours and 27 to 48 hours after traumatization. Of 100 mice injected with the drug at a dose level of 4, 8, or 16 μg per g, in distilled water or isotonic saline, immediately after traumatization, 15 survived to 24 and also to 48 hours. No matter what procedure was tested, the drug was completely ineffective in promoting survival when administered immediately *after* heat traumatization.

Discussion. In the present experiments, variation in type of trauma used and time of administration of chlorpromazine in relation to induction of trauma resulted in considerable variation in the activity of this drug in counteracting: (a) trauma induced hemoconcentration, and (b) trauma induced death. However, (a) and (b) activities of chlorpromazine ran roughly parallel. Since hemoconcentration associated with shock is indicative of decreased circulating blood volume, chlorpromazine probably protects mice against trauma induced death, in part at least, by minimizing a trauma induced decrease in circulating blood volume. Chlorpromazine is effective in increasing the likelihood of survival of rats after tumbling trauma(3) or of mice after heat trauma (this paper) *only* if administered prior to traumatization. On the other hand, the drug increased likelihood of survival whether administered before or some time after severe hemorrhage in the dog(2, 3), or before or shortly after application of hind leg ligatures in tourniquet traumatization of the mouse (this paper). The above relations are considered important in relation to possible clinical use of this or related drugs in traumatic or hemorrhagic shock. Wiggers(9) has reviewed the considerable body of evidence that toxic substances are formed in tissue areas subjected to severe trauma and/or anoxia associated with hemorrhage or circulatory occlusion, and that these toxic substances play an important role in the development of irreversibility of the shock syndrome. It is likely that such substances are formed rapidly in association with tumbling or heat trauma, more slowly in association with severe hemorrhage. Toxic substances formed in tissue areas deprived of circulation by use of tourniquets could not reach the general body circulation until after removal of the tourniquets. It is therefore postulated here as a working hypothesis that chlorpromazine, when administered a sufficient period of time before removal of hind leg tourniquets, decreases the reactivity of the cardiovascular system of the mouse to toxic substances formed in the hind legs during occlusion of circulation to such a degree as to prevent development of irreversibility of the tourniquet trauma shock syndrome in some mice.

Summary. Chlorpromazine appreciably counteracted tourniquet trauma induced

hemoconcentration and death when administered either before or after *placement* of hind leg ligatures, but not after *removal* of these ligatures. Maximal counteraction was secured by administering the drug before or immediately after placement of the ligatures. Chlorpromazine was effective in appreciably counteracting heat trauma induced hemoconcentration and death only if administered prior to traumatization.

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Production of Aldosterone by Rat Adrenal Glands *in vitro*.*† (22635)

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Rat adrenal glands have been shown to produce corticosteroids *in vitro*(1,2,3) and a stimulation of this production by the addition of ACTH to the incubation medium had been demonstrated(1). Small amounts of aldosterone were found by Wettstein and Anner(4) in the incubation medium of beef and hog adrenals, and by Rosenfeld(5) and associates in the perfusion medium of beef adrenals. The present work shows that the incubation medium from rat adrenals contains a sodium-retaining substance with the properties of aldosterone and describes the results of experiments performed to investigate some of the factors which may affect its secretion.

Methods. In each experiment adrenal glands were obtained from 40 to 90 male rats weighing between 170 to 180 g. These animals were subsequently used for bioassay purposes. The glands were freed of fat and quartered. One quarter of each adrenal was placed in each of 4 flasks containing 1.5 ml per 25 mg of fresh tissue of Krebs-Ringer-bicarbonate solution with added glucose (200 mg%)(6). Thus the 4 flasks contained an equal amount of tissue from each rat in the group. The incubation of the tissue was carried out in a water bath at 38°C. After the first hour the medium was poured off and replaced by an equal quantity of the same solution. The incubation was allowed to continue for an additional 2 hours. A gentle stream of 95% O_2 - 5% CO_2 was passed through the medium continuously. At the end of this period the medium was decanted. A 0.5 ml aliquot was extracted with methylene chloride for determination of the total Δ^4 3-ketosteroid content, according to the method of Saffran and Schally(6). The rest of the medium was extracted with 3.5 times its volume of redistilled chloroform in glass stoppered cylinders. The two phases were separated by centrifugation and the chloroform layer was carefully transferred with a syringe and a long needle into a round bottomed flask and evaporated to dryness *in vacuo* at a water bath temperature of 50°C.

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In earlier experiments this crude extract was assayed directly for sodium-retaining activity. In later studies it was subjected to paper partition chromatography in the benzene-aqueous methanol system of Bush(7), run at room temperature for 4½ hours. The aldosterone band was subsequently bioassayed. Detection and partial characterization of the material after chromatography was done by direct visualization and/or contact photography under ultra violet light; by scanning of the paper strip in a Beckman DU spectrophotometer at 2400 Å; by soda fluorescence, blue tetrazolium and triphenyltetrazolium reactions on the chromatogram; by mixed chromatogram with crystalline compounds and absorption spectrum in sulfuric acid solution. In certain experiments, paper chromatographic separation of aldosterone was followed by spectrophotometric determination of the eluate at 2400 Å. This procedure was made possible without further paper chromatographic purification of aldosterone, since cortisone, which in the benzene aqueous methanol system has a mobility closely related to aldosterone, was not found in the incubation media of rat adrenals. Furthermore after such a chromatographic separation, in 14 experiments with and without ACTH the amounts of aldosterone, as determined both by bioassay and by spectrophotometry, were highly significantly correlated. Sodium-retaining activity was assayed on adrenalectomized rats according to a modification of the method of Singer and Venning(8).

Results. The Na retaining activity of the crude chloroform extracts of rat adrenal incubates varies from 0.27 to 0.73 μg equivalent of aldosterone per 100 mg of tissue per hour of incubation (Mean of 6 experiments, 0.46 ± 0.09).

Paper partition chromatography of these extracts revealed the presence of several ultraviolet-absorbing compounds. Of these, one was tentatively identified as corticosterone by mixed chromatogram, sulfuric acid chromogen, maximum ultraviolet absorption at 2400 Å in methanol and reduction of the tetrazolium reagents. Corticosterone represents about 45 to 50% of the Δ^4 3-ketonic compounds produced *in vitro* by rat adrenals.

A strong sodium-retaining activity was found by biological assay of an ultraviolet absorbing α-ketolic material recovered from the area between the bottom of cortisol and the top of cortisone as defined by a reference strip. This material yielded 0.31 to 0.64 μg equivalent of aldosterone per 100 mg of tissue per hour of incubation (mean of 12 experiments, 0.47 ± 0.03). The sodium-retaining activity separated both from cortisol and cortisone on mixed chromatography and on bioassay showed a dose-response relationship with a slope identical with that of aldosterone. That the sodium-retaining activity was limited to the chromatographic area as defined above, was demonstrated by the fact that eluates of immediately adjacent areas showed no activity on bioassay at level up to 4 times the equivalent dosage. From these findings, it may be concluded with a fair degree of certainty that the ultraviolet-absorbing α-ketolic material under study is aldosterone.

The other compounds present on the chromatograms did not show noticeable sodium-retaining potency in the assay. However, the presence of traces of desoxycorticosterone which, in the chromatographic system used would run close to the solvent front, has not been eliminated.

Action of ACTH. In 4 out of 5 studies the addition of ACTH, 1 to 100 milli-units/100 mg of tissue, significantly increased the

TABLE IA. Sodium Retaining Activity of Incubation Media of Rat Adrenal Glands after Addition of ACTH. (Crude fraction-biological assay.)

ACTH, milliunits per 100 mg tissue	Na retaining activity Aldosterone eqv., μg/100 mg tissue/hr Assay 1	Assay 2	Δ^4-3-keto-steroids, μg/100 mg tissue/hr
Control	.30	.50	10.3
1	1.25 ($<$.02)	1.10 ($<$.01)	10.8
10	1.09 ($<$.02)	1.00 ($\simeq$.02)	14.8
100	1.52 ($<$.01)	1.50 ($=$.001)	28.2
Control	.14	.21	
100	.61 ($<$.02)	.40 ($>$.1)	

Sodium-retaining activity of each medium with added ACTH was compared to that of control medium without ACTH and its degree of significance was calculated by Student's t test. Value of P is shown in parentheses.

TABLE IB. Action of ACTH on Production of Aldosterone by Rat Adrenal Glands Incubated *In Vitro*.*

ACTH, milliunits/100 mg of tissue	Aldosterone, μg/100 mg of tissue/hr Control	Aldosterone, μg/100 mg of tissue/hr ACTH added	Corticosterone, μg/100 mg of tissue/hr Control	Corticosterone, μg/100 mg of tissue/hr ACTH added
100‡	.63	.79	—	5.00
"	.50	.63	2.42	4.60
100§	.54	.71	—	—
"	.52	.59	1.74	5.19
"	.62	.76	2.23	3.55
200‡	.32	.51	2.56	6.94
Mean	.52 ± .05†	.67 ± .04	2.24 ± .18†	5.07 ± .55
	Mean diff. = .14 ± .02 Significant at $P < .001$		Mean diff. = 2.83 ± .46 Significant at $P < .01$	

* Quantitative determination of aldosterone in Beckman spectrophotometer at 240 mu.
† Stand. error of mean.
‡ Acton X Nordic Biochemical Labs. Lot 54009.
§ ACTH Connaught Lot 8-3.

sodium-retaining activity of the crude chloroform extract of the incubation media. However, a dose-response relationship was not established between the amount of ACTH and that of the sodium-retaining material (Table 1A). Comparing the action of ACTH on production of the sodium-retaining material and on that of the total Δ^4 3-ketosteroids, it was observed that production of the former was enhanced by a dose of 1 milliunit whereas that of the corticoids increased only at a dose level of 10 and 100 milliunits. Further experiments are needed to establish reproducibility of the response of the sodium-retaining substance to the dose of 1 milliunit of ACTH. To exclude the stimulatory effect of ACTH on other possible Na-retaining substances similar studies were performed with paper chromatographic separation of aldosterone and its quantitative measurement by spectrophotometry (Table IB). They showed that at the dose level of 100 to 200 milliunits/100 mg of tissue, ACTH has a small but consistent effect on the production of aldosterone *in vitro* (mean difference of 6 studies, 0.14 ± 0.02 μg of aldosterone/100 mg of tissue/hr of incubation. Significant at $P < .001$). The relatively greater increase in sodium-retaining activity of the crude chloroform extracts after ACTH stimulation of the glands is probably due to a sodium-retaining substance or substances other than aldosterone.

Action of growth hormone. Two preparations of growth hormone were tested for their ability to affect the sodium-retaining material released by rat adrenals *in vitro*. The most purified preparation (C. H. Li's Growth Hormone) was without detectable effect at the dose level of 10 and 100 μg/mg of tissue. At the 250 μg dose level, the other preparation (Horner) sensibly increased both the Δ^4 3-ketosteroid content and the biological sodium-retaining potency of the incubation medium, indicating that it was probably contaminated with ACTH (Table II).

Effect of altering Na/K ratio of the incubation medium. Two series of incubations were carried out after alteration of the Krebs-

TABLE II. Sodium Retaining Activity of Incubation Media of Rat Adrenal Glands after Addition of Growth Hormone. (Crude fraction-biological assay.)

Growth hormone, μg/100 mg tissue	Na retaining activity: Aldosterone eqv., μg/100 mg of tissue/hr	Δ^4-3-ketosteroids, μg/100 mg of tissue/hr
Control	.41	10.8
10*	.50	11.3
100*	.45	12.3
Control	.37	
10*	.31	
100*	.28	
Control	.28	10.1
250†	.44‡	18.1

* Growth hormone prepared by Dr. C. H. Li and kindly given to us by Dr. H. Selye.
† Somatrofin, Horner Laboratories, Lot No. P.R. 3.
‡ Sodium retention compared to the control is significant at $P < .01$.

TABLE III A. Effect of Variation of Na/K Ratio of Incubation Media on Production of Sodium Retaining Material and Aldosterone by Rat Adrenal Glands.

A: Crude fraction biological assay

	Na retaining activity:	
Na/K*	Aldosterone eqv., μg/100 mg of tissue/hr	Δ^4-3-ketosteroids, μg/100 mg of tissue/hr
24.3	.24	11.8
16.3	.14	14.0
4.0	.32	13.3
17.9	.65 (<.1)	14.5
24.3	.11	9.3
16.3	.46 (<.001)	13.1
4.0	.74 (<.001)	11.4
17.9	.28 (<.01)	12.6

TABLE III B.

B: Paper chromatography

Na/K*	Aldosterone,† μg/100 mg tissue/hr	Corticosterone, μg/100 mg tissue/hr
24.3	.97	4.0
16.3	1.32	5.6
24.3	.69	4.8
4.0	1.02	5.4
24.3	.82	6.3
16.3	1.23	5.7

Mean diff.: .36 ± .025
Significant at $P < .01$

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* The following concentrations of Na and K (in meg/l of medium) have been used: (Na: 143.4, K: 5.9) Na/K = 24.3. (Na: 138.6, K: 8.6) Na/K = 16.1. (Na: 119.8, K: 29.6) Na/K = 4.0. (Na: 96.5, K: 5.38) Na/K = 17.9.

† Quantitative determination of aldosterone in Beckman spectrophotometer at 240 mu.

Ringer-bicarbonate medium from its original Na/K ratio of 24 to a ratio of 16 and 4 by lowering the Na and increasing the K concentrations. In 2 cases the Na concentration only was decreased, the K concentration remaining about the same as that of the control (Table IIIA). Under these conditions the sodium-retaining potency of the crude fraction of the altered media did not significantly differ from that of the control in the first series of experiments, but did so in the second. However in this second series, the control value of 0.11 μg equivalent of aldosterone is unusually low as compared to the range previously stated for the crude fraction (0.27 to 0.79 μg/100 mg of tissue/hour). More consistent results were obtained after paper chromatographic separation of aldosterone followed by its spectrophotometric determination (Table IIIB). In 3 studies, the production of aldosterone was found to be increased in the media having a Na/K of 16 and 4 respectively. The mean difference of 0.36 ± 0.025 μg of aldosterone/100 mg of tissue/hour is significant at $P < .01$.

Discussion. In the present study bioassays of the crude chloroform extracts of the incubation media were performed only as a guide to production of sodium-retaining substance or substances by the rat adrenals submitted *in vitro* to various stimuli. The inconsistent values sometimes obtained by bioassays of these crude chloroform extracts are not unexpected since such extracts probably represent a mixture of substances which individually might have widely different or even antagonistic effects on sodium retention. Therefore, the biological activity of the crude fractions cannot be compared rigidly with the quantitative measurement of aldosterone separated by paper chromatography and subsequently measured by bioassay or spectrophotometry. Recent reports have shown that aldosterone or a potent electrolyte-regulating substance, in all probability identical with aldosterone, is produced by incubation of beef and hog adrenal homogenates(4) as well as by perfusion of calf adrenal(5). The present data demonstrate that under *in vitro* conditions the yield of aldosterone by rat adrenals is much greater per unit of weight. Studies on hypophysectomized animals have suggested that the pituitary may play a role in the secretion of aldosterone since the amount of the hormone recovered from adrenal vein blood of hypophysectomized rats(9) and dogs(10) was found to be lower than in normal controls. Our findings tend to support this view since a slight but consistent increase in aldosterone production was obtained *in vitro* by addition of 2 different ACTH preparations to the incubation media. However, this effect may be due to contamination of the ACTH preparations with some other pituitary hormone. On the other hand, growth hormone is unlikely to enhance the secretion of aldosterone by a direct effect. Similar conclusions have been reached by Rosenfeld and collaborators after perfusion of calf adrenals with ACTH and with growth hormone(5). Recent *in vivo* studies have indicated the probability that the main stimulus of aldosterone secretion is directly or indirectly related to certain changes of sodium, potassium, and water metabolism. Although the results presented here show that alteration of the Na/K ratio of the incubation media is accompanied by an increase in aldosterone secretion, the physiological significance of these experiments seems to be limited by the following considerations: 1) They do not disclose whether alteration of Na or K concentrations or both is the effective stimulus for the observed increase of aldosterone secretion. 2) This increase is not very striking, leaving open the possibility that the alteration of Na and K concentrations *in vivo* may exert major effects by an indirect action which would not be demonstrable by the study of isolated glands.

Conclusion. Rat adrenal glands incubated *in vitro* released a sodium-retaining substance with the properties of aldosterone. Purified growth hormone does not seem to increase secretion of sodium-retaining activity. Two ACTH preparations caused a slight but significant increase of aldosterone production *in vitro*. Alteration of the Na/K ratio of the incubation media, by lowering the Na and increasing the K concentration, produces a moderate enhancement of aldosterone secretion.

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Strontium-Calcium Discrimination Factors in the Rat. (22636)

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The behavior of strontium in the animal body has become of particular interest in relation to possible hazards from atomic fission debris. Contamination of the food chain by fallout material may create a situation in which the calcium of the dietary becomes associated with essentially carrier-free radiostrontium. The body burden of radiostrontium, in terms of body calcium, attained after ingestion over long periods of time will be governed by the Sr*/Ca of the intake and by the over-all differentiation between strontium and calcium that may take place in such processes as primary absorption from the tract; incorporation into new bone; incorporation into pre-existing bone; renal excretion; endogenous excretion into the gastrointestinal tract; and reabsorption from the tract.

Comar, Whitney, and Lengemann(1) showed that growing rats utilized dietary calcium in a commercial ration for bone growth by a factor of 3.6 over carrier-free Sr 90 that had been incorporated into the feed, *i.e.*, the Sr 90/Ca ratio of the diet divided by the Sr 90/Ca ratio of the bones equaled 3.6. These rats had been raised for one or 2 generations on a diet in which the Sr 90 to calcium ratio was maintained at a constant level so that their bones were formed entirely from the Ca-Sr 90 of the "spiked" diet. This finding was confirmed by Alexander, Nusbaum, and MacDonald(2) who estimated stable strontium-calcium ratios in the bones and diets of experimental animals.

The present paper is concerned with (a) development of shorter methods for comparison of calcium and strontium metabolism by the use of double tracer techniques; (b) demonstration of the effect of normal types of diets on the relative metabolism of calcium and strontium; and (c) estimation of the extent to which the individual processes in the body differentiate between strontium and calcium.

Methods. Previous work(1) had shown that Ca 45 incorporated into the diet was utilized to the same extent as the dietary calcium. Therefore, it was considered that the behavior of Ca 45 and Sr 89 administered in the diet for a matter of weeks would give a true picture of the behavior of dietary calcium and incorporated radiostrontium. This would avoid the long feeding period necessary to obtain animals whose bones had been entirely formed from the "spiked" diet. Three groups of rats were raised on diets as follows: *Milk*—evaporated milk was supplemented to contain 8.5 mg $MnCl_2 \cdot 4H_2O$, 4.2 mg $CuSO_4$, 27 mg $FeSO_4 \cdot 7H_2O$, 14 mg KI and 4 g yeast extract (Difco) per liter of reconstituted milk; *Milk and Corn*—in addition to the milk supplemented as above, each animal received 1 g of ground yellow corn per day; *Commercial*—Rockland rat pellets. The animals on the "Milk" and "Commercial" diets were on experiment between 30 and 50 days of age, whereas the other group was studied between 21 and 81 days of age (Table I). The animals on the "Commercial" diet received the Ca 45 and Sr 89 (in constant ratio) in their drinking water; the other groups received the tracers in the milk. The radioactivity dosage amounted to about 0.4 μc of Ca 45 and 0.2 μc of Sr 89 per animal per day; relatively high specific activity preparations were used. During the latter part of the experiment, the animals were placed in metabolism cages for quantitative collections of feces and urine. At termination, representative animals were killed to provide samples of blood and bone (entire femurs). The 2 radioisotopes were measured by the usual methods for radioassay of mixtures based on differential absorption (3,4). Twelve to 15 rats had been started on each diet. However, the analytical results were in sufficient agreement that only 3 to 4 animals were used and the remainder were carried for another study.

Expression. In the original paper on this subject(1), the results were discussed in terms of preferential utilization of calcium over strontium as already mentioned. Alexander *et al.*(2) then proposed the term "bone retention factor", which is essentially the reciprocal of the preferential calcium utilization. To denote the individual tissues, excretions, or physiological processes involved, it is necessary to have broader terminology. We are therefore proposing the term "Strontium-Calcium Observed Ratio" (OR) to denote the actual differentiation in tissues or excreta and defined as follows:

$$OR_{sample\text{-}precursor} = \frac{Sr/Ca \text{ of sample}}{Sr/Ca \text{ of precursor}} \qquad (1)$$

This observed ratio (OR) does not imply that the tissue in question has exerted any action in causing the discrimination.

To denote the discrimination that is produced by a given physiological process, we are proposing the term "Strontium-Calcium Discrimination Factor" (DF). By definition:

$$OR = (DF_1)\ (DF_2)\ (DF_3)\ \ldots\ (DF_n) \qquad (2)$$

Thus, for example, the symbol $DF_{urinary}$ is used to designate the contribution to the overall discrimination in the body that resulted from differential handling of the two elements by the kidney.

It is noted that OR and DF as defined here are the conceptual inverse of the preferential calcium utilization, and describe strontium behavior in terms of calcium. They are thus similar to the retention factor; when OR or DF is less than 1, this means that the strontium is discriminated against in favor of calcium.

Data and Calculations: Table I presents data for (a) OR values as measured for bone, blood, feces, and urine in double tracer experiments under conditions of daily intake; (b)

TABLE I. Strontium-Calcium Observed Ratios and Percentage Excretion in the Rat. (Values represent mean ± stand. error of mean.)

Diet	Milk	Milk and corn	Commercial
No. of animals	4	3	4
Age at start and end of study, days	30–50	21–81	30–50
$OR_{bone\text{-}diet}$*	.57± .02	.45± .01	.27± .01
$OR_{retained\text{-}diet}$†	.57	.56	.30
$OR_{blood\text{-}diet}$	.46± .04	.43± .08	.21± .04
$OR_{urine\text{-}}$ "	1.99± .01	1.47± .03	1.1 ± .03
$OR_{feces\text{-}}$ "	1.41± .08	1.33± .05	1.27± .03
% excretion‡			
Ca 45 in urine	4.5 ±1.1	5.7 ± .6	1.8 ± .3
Sr 89 " "	8.7 ±1.9	8.4 ± .6	1.9 ± .2
Ca 45 in feces	44 ±2	50 ±3	71 ±6
Sr 89 " "	62 ±4	67 ±2	90 ±8

* $OR_{bone\text{-}diet} = \frac{Sr^*/Ca^* \text{ in bone}}{Sr^*/Ca^* \text{ in diet}}$.

† Calculated by difference from excretion values.

‡ $\frac{\text{Excretion during last 4 days of exp.}}{\text{Intake during last 4 days of exp.}} \times 100$.

OR for the entire body as calculated by difference from excretion values; and (c) percentage excretion of Ca 45 and Sr 89 under conditions of daily intake.

As will be discussed later, there appear to be only 2 major discriminatory processes in the rat: absorptive and urinary. Under these conditions, and with the assumption that the urinary excretion of each element is proportional to the amount absorbed, the following equations are easily derived for calculation of the Strontium-Calcium Discrimination Factors:

$$DF_{absorptive} = \frac{100 - \%\ Sr\ 89 \text{ in feces}}{100 - \%\ Ca\ 45 \text{ in feces}} \qquad (3)$$

$$DF_{urinary} = \frac{OR_{retained\text{-}diet}}{DF_{absorptive}} \qquad (4)$$

Table II presents the DF values as calculated in this way as well as the comparison of blood and urine values to indicate the relative renal clearances of calcium and strontium. It will be recognized that the DF values as estimated in this way represent the over-all contribution to the discrimination, and do not take into account such matters as the change in Sr/Ca ratio as the food passes through the gastro-intestinal tract.

Discussion. It is first noted that the $OR_{bone\text{-}diet}$ value of 0.27 reported for the commercial diet in Table I is in excellent agreement with previous values of 0.28(1) and 0.27(2) determined by other methods. Thus, any of the following methods should be valid for prediction of naturally occurring Sr/Ca ratios or Sr*/Ca ratios when the intake is known: (a) analysis and radioassay of bone formed entirely from feed with constant ratio of added Sr* to naturally occurring calcium (1); (b) analysis of natural strontium-calcium ratios in a given diet and bones formed from this diet(2); and (c) radioassay of tissues or excretions from animals on a daily intake of Sr* and Ca* for a matter of weeks. The last method will usually be the most convenient from the experimental standpoint.

The $OR_{retained\text{-}diet}$ values as calculated from the balance results are in fair agreement with the $OR_{bone\text{-}diet}$ values. Differences are probably due to inaccuracies in collection of excreta and analysis or less likely to the bone not having a Sr*/Ca* ratio representative of the entire body. The higher $OR_{bone\text{-}diet}$ values of 0.59 and 0.42 for the animals on the milk-containing diets were anticipated and agree with the findings of Wasserman *et al.*(3), who showed that substances such as lactose and l-lysine that increased calcium absorption as does milk, were even more effective in promoting Sr 89 absorption than Ca 45 absorption. This emphasizes that the nature of the diet is important in governing not only the extent of alkaline-earth absorption, but also the discrimination that occurs.

Processes with minor discrimination: It is convenient first to consider those physiological processes by which little or no differentiation occurs. Any discrimination in movement of calcium and strontium from blood to skeleton

TABLE II. Strontium-Calcium Discrimination Factors in the Rat.

Diet	Milk	Milk and corn	Commercial
$\frac{OR_{urine\text{-}diet}}{OR_{blood\text{-}diet}}$	4.3	3.4	5.2
$DF_{urinary}$	.84	.84	.88
$DF_{absorptive}$	.68	.66	.34
$OR_{retained} = (DF_{urinary})(DF_{absorptive})$	.57	.56	.30

can be estimated by comparison of $OR_{blood\text{-}diet}$ and $OR_{bone\text{-}diet}$ values. Table I shows that the strontium rather than being discriminated against was, if anything, preferentially utilized by bone ($0.02 < p < 0.05$). This may be of interest in connection with early observations on strontium retention that led to strontium therapy for osteoporosis(5). For purposes of calculation of over-all discrimination, however, it can be assumed that movement from blood to skeleton does not contribute significantly. This conclusion was also reached by Bauer *et al.*(6). The relatively large uncertainties in the $OR_{blood\text{-}diet}$ values resulted from the low levels of radioactivity present in the blood in these studies; these low levels led to counting difficulties.

It also appears that excretion of calcium and strontium from the blood into the gastrointestinal tract via the bile, pancreatic juices, and secretions of intestinal mucosa would not contribute in a major way to the over-all discrimination. Greenberg and Troescher(7) found little difference in the biliary excretion and total fecal excretion following intraperitoneal injection of Ca 45 and Sr 89 into rats. From short term experiments with dogs, Singer *et al.** found about twice as much Sr* was secreted in the bile as Ca*; the total excretion of Sr* into the gastrointestinal tract, however, exceeded that of Ca* by only about 20%. Also, the percentage of parenterally administered Sr* and Ca* that reaches the intestinal tract is relatively small, 7 to 20%. In the present paper, no attempt is made to distinguish between endogenous and unabsorbed Sr* and Ca* in the fecal excretion values.

Processes with major discrimination: The renal clearance of strontium was 3 to 5 times that of calcium as indicated by the ratios of $OR_{urine\text{-}diet}$ and $OR_{blood\text{-}diet}$ values in Table II. This general behavior has also been observed in man, dog, and bovine(8,9,10). It is also apparent, however, from Table II that the differential absorption contributed more to the over-all discrimination than did the differential urinary excretion. This was more pronounced in the rats on the commercial diet ($DF_{absorptive} = 0.34 : DF_{urinary} = 0.86$). The contribution of the urinary discrimination was essentially the same in all 3 groups. The $DF_{absorptive}$, however, was about 0.67 for the milk-containing diet. These differences are of the same order as reported for the effects of lactose on relative strontium and calcium absorption.

As an independent confirmation of the ($DF_{urinary}$) value, an experiment was done in which rats on a commercial diet were injected intraperitoneally with Ca 45 and Sr 89 and the excretions studied. Under these conditions $OR_{retained} = 0.85$, $DF_{urinary} = 0.89$ and $DF_{fecal\ excretion} = 0.96$. The discrimination factor due to fecal excretion resulted both from differential endogenous excretion and differential reabsorption from the gastrointestinal tract. As expected the contribution from both of these processes was small. It is of interest to note the agreement between the $DF_{urinary}$ value as obtained in this experiment, and that in Table II from the continuous feeding study.

Comparison of discrimination among species is somewhat difficult because of the variables involved, such as age and dietary intake. Harrison *et al.*(8), however, reported that adult man receiving a somewhat higher stable strontium intake than normal, absorbed calcium about twice as much as strontium. For cattle, it appears that absorptive discrimination is small(10). Values of $OR_{bone\text{-}diet}$ for laboratory animals on commercial diets were reported as follows: mice, 0.35; rats, 0.27; and guinea pigs, 0.22. The ratios for Nevada desert animals were jack-rabbits, 0.20; cottontail rabbits, 0.22; and kangaroo rats, 0.16(2).

The principles as presented here can also be used in studies where lactation or deposition in the fetus provide additional discriminatory processes.

Summary. The term "Strontium-Calcium Observed Ratio" (OR) is proposed and defined to designate the over-all discrimination that occurs in movement of the 2 elements from one phase to another in a biological system. The term "Strontium-Calcium Discrimination Factor" (DF) is proposed and defined

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* Singer, L., Maqsood, M., Medlen, A. B., and Comar, C. L., unpublished results.

to characterize the contribution of individual processes to the over-all discrimination. Double tracer studies of Ca* and Sr* ingestion over a 20-day period gave the same OR values as methods based on lifetime feeding. Absorption from the tract and urinary excretion were the major processes by means of which the rat discriminated against dietary strontium in favor of calcium. There is evidence of slight strontium preference in movement of the two elements from blood to bone. Excretion into the gastrointestinal tract did not contribute significantly to the over-all discrimination. Rats on a nonmilk diet ($OR_{\text{bone-diet}} = 0.27$) discriminated more against strontium than did those on a milk diet ($OR_{\text{bone-diet}} = 0.57$). This behavior was related to the absorptive process.

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Effect of Digoxin on Cardiac Glycogen of the Rat.* (22637)

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Non-toxic doses of cardiac glycosides have been reported to increase cardiac glycogen (1,2,3). On the other hand, the data of Bomskov and Kaulla(4) and Liebig(5) indicate non-toxic doses decreased the glycogen level of the heart, while the results of Kimura (6) showed digotoxin to be without effect. Toxic doses of the cardiac glycosides have been reported to cause increased glycogen in the heart of the pigeon(7) and in the rat doses of 3.4 mg of digitoxin per kilo body weight resulted in an increase of the cardiac glycogen(4). Nevertheless, depletion of cardiac glycogen in animals manifesting signs of cardiac glycoside poisoning has been reported by Cherkes(2) and Haendel and Munilla(8).

The recent report of Bloom, Lewis, Schumpert, and Shen(9) that the glycogen of tissues can be divided into acid soluble and acid insoluble fractions which maintain a reasonably constant ratio to each other and the report of Russell and Bloom(10) that most physiological changes in the tissue glycogen take place in the acid soluble (labile) fraction while the acid insoluble (protein bound) remains relatively constant suggested that some of the equivocal results of the digitalis studies could be resolved by a consideration of the effects of cardiac glycosides on each of the two glycogen fractions.

Methods. Female rats, of various ages, were injected subcutaneously with 0.2 mg/kg of digoxin suspended in distilled water. With the exception of the newborn animals, the rats were injected daily for 5 days and sacrificed 5 hours after the last injection on the 5th day. The newborn animals received one

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* This study was supported by a grant from the American Heart Association.

injection and were sacrificed 5 hours later. Animals receiving a toxic dose of digoxin, were given 100 mg of the drug and sacrificed 5 hours later. All animals were non-fasted and had been fed Purina Laboratory chow supplemented by fresh greens once a week up to the time of sacrifice. At the time of the sacrifice the animals were given an intraperitoneal injection of sodium pentobarbital (40 mg/kg). The newborn and 10-day-old rats were decapitated. With a sharp scalpel, a single incision was made through the rib cage, the heart grasped, the ventricle snipped and quickly placed between 2 slabs of dry ice. The frozen button of tissue was removed and divided into 2 parts. It was necessary to pool from 3 to 5 hearts of animals in the newborn and 10-day age groups in order to obtain sufficient tissue for analysis. One part of the frozen tissue was weighed and placed in 2 cc of 30% KOH. After tissue solution, the glycogen was precipitated by adding 1.1 to 1.2 vol. of 95% alcohol(11). The other part was weighed and placed in 5 cc of ice cold 10% trichloroacetic acid contained in a glass tissue homogenizer set in cracked ice. After homogenization in the cold room, the content of the homogenizer tube was transferred to a centrifuge tube, the homogenizer washed with an additional 5 cc of ice cold 10% trichloroacetic acid and the wash added to the centrifuge tube. After centrifugation, the clear supernatant was decanted and the glycogen precipitated from the supernatant with 2 vol. of ice cold 95% alcohol. The glycogen was allowed to precipitate in the cold over night, and then centrifuged. The glycogen from both procedures was hydrolyzed by addition of 2 cc of 0.6 N HCl and heating in a boiling water bath for $2\frac{1}{2}$ hours. The acid hydrolysate was neutralized to phenolphthalein, made up to an appropriate volume with water and 2 cc taken for glucose determination by the method of Benedict(12).

Results. The results presented in Table I show that in the normal animal the trichloroacetic acid soluble (labile) fraction of cardiac glycogen, remains reasonably stable from birth through 360 days of age with a rather abrupt decrease in labile glycogen in the 100-

TABLE I. Cardiac Glycogen.

	Controls					Digoxin treated, .2 mg/kg subcut. daily for 5 days*				
			Glycogen in mg/100 g fresh ventricular tissue					Glycogen in mg/100 g fresh ventricular tissue		
Age in days	No. animals	Body wt, g	Total	Acid sol.	Acid insol.	No. animals	Body wt, g	Total	Acid sol.	Acid insol.
Birth	8†	6.5	221 ± 7‡	173 ± 7	49 ± 8	8†	6.5	249 ± 9	183 ± 6	66 ± 8
10	5†	16 ± 2	259 ± 5	159 ± 3	100 ± 5	5†	14 ± 1	289 ± 5	160 ± 5	129 ± 7
30	8	92 ± 2	367 ± 17	153 ± 11	214 ± 20	8	92 ± 2	367 ± 13	144 ± 7	223 ± 12
100	11	177 ± 10	420 ± 12	93 ± 7	327 ± 15	10	169 ± 9	320 ± 21	41 ± 4	279 ± 18
360	11	241 ± 7	462 ± 21	149 ± 9	313 ± 23	12	240 ± 5	296 ± 27	86 ± 16	210 ± 30
630	7	411 ± 14	363 ± 16	115 ± 6	249 ± 12	7	414 ± 12	378 ± 10	42 ± 7	336 ± 14
								100 mg subcut.§		
						5	170 ± 11	501 ± 22	101 ± 11	400 ± 19‖
						5	247 ± 7	700 ± 20	256 ± 14	444 ± 22¶

* Rats at birth given 1 inj. and sacrificed 5 hr later. † No. of pooled samples. ‡ $\sqrt{\frac{\Sigma d^2}{N(N-1)}}$. § Single inj. and sacrificed 5 hr later.

‖ Controls for this group are the 100-day-old. ¶ Controls for this group are the 360-day-old.

day age group, and a significant fall in the 630-day-old rats. The trichloroacetic acid insoluble (bound) fraction shows a steady rise from birth through 100 days of age, a stable level from the 100 through the 360-day age group and a decline in the older 630-day age group.

Effect of Digoxin on cardiac glycogen. Table I shows that digoxin has a varied effect on the cardiac glycogen of the rat when injected before 30 days of age. When rats at birth are treated with the drug, there is a slight increase in total glycogen. The significance of this rise becomes questionable when it is seen to be due to the additive effect of a small non-significant increase in both the labile and bound fractions. In the 10-day age group a 12% increase in total glycogen resulting from the digoxin injection was due entirely to the 29% increase which occurred in bound glycogen fraction. The labile fraction showed no change from the control. In 30-day-old rats digoxin gave no evidence of effect on cardiac glycogen.

At 100 days the heart responded to digoxin with a 24% decrease in total glycogen. This fall, when considered in terms of absolute amounts, occurred in both the labile (52 mg depression) and bound (48 mg decrease) fraction. The labile fraction fell 56% from the control value. The 15% change in the bound fraction, although statistically of doubtful significance ($P = 0.10$-0.05), is suggestive of the onset of bound glycogen depression evident in the next age group studied. In 360-day-old rats, digoxin depressed the total cardiac glycogen, which fell from a control level of 462 mg per 100 g tissue to 296 or a decrease of 36%. Both glycogen fractions contributed to the decrease. The acid soluble (labile) fraction was decreased 43% while the acid insoluble (bound) fraction fell 33%. In animals at 630 days of age, digoxin caused no change in total glycogen. However, this negative effect was due to a 55% increase in bound glycogen, accompanied by a 64% decrease in labile glycogen.

Effect of toxic dose of digoxin on cardiac glycogen. The 100- and 360-day age groups were selected to note the toxic effect of 100 mg of digoxin on cardiac glycogen. The drug was administered in one dose and the animals sacrificed 5 hours later. Both age groups showed a rise in total and bound glycogen with this toxic dose. The labile fraction rose 72% in the 360-day age group while the 100-day age group showed no change from the control.

Discussion. These results suggest that the age of the animal has an influence on response of the cardiac glycogen stores to digoxin treatment. Rats of 30 days of age or less did not respond to digoxin or in some cases as the bound fraction of the 10-day age group, with a slight increase. Digoxin consistently caused a decrease in the acid soluble fraction of glycogen in the hearts of all rats of 100 days or older. Russell and Bloom(10) in commenting upon previous work and adding results of their own have suggested that the labile glycogen is the more mobile portion physiologically and seems to be associated with the immediate functional demands made upon the tissue. This is of interest, in view of Wollenberger's(13) comments that the effect of the cardiac glycosides do not appear to be related to the energy yielding processes of muscle. The age groups represented by 100- and 360-day-old rats respond to digoxin with a decrease in total glycogen. In the 100-day age group the fall in total glycogen is due to an equal, in absolute amounts, decrease in the labile and bound fraction The digoxin depression in the 360 age group is predominantly due to a decrease in the bound fraction of glycogen. The ages 100 and 360 days fall within the period of sexual maturity and activity which implies that the sex hormones may alter the digoxin response of labile and bound glycogen. This problem is under investigation.

The values on cardiac glycogen here reported are higher than the results reported elsewhere(10,14,15) This may be attributable in part to a sex difference; preliminary observations in our laboratory suggest that this may be true at least in regard to estrogens. Leonard(16) reported that estradiol increased glycogen content of skeletal muscle in spayed females. The higher values reported here may be due to the Benedict method for glucose which is sensitive to nonfermentable reducing substances occurring in acid hydrolysates of tissues. However, values obtained in our laboratory for liver glycogen using the Benedict method for glucose agree with those reported by Russell and Bloom (15), using the anthrone method. In the instance where approximate ages are mentioned for the male rats used by Bloom and Russell (14) age (from 3-4 mo. to 6-8 mo.) increased both the labile and bound glycogen whereas in our series of females the labile fraction remained relatively constant from birth through 360 days of age with a considerable fall occurring in the 100-day age group. Nevertheless, results are consistent with the rise due to age reported by Bloom and Russell(14) if their 3-4 mo. old series is considered comparable to our 100-day age group.

Several observations may have a bearing on the increase in cardiac glycogen observed with toxic doses of digoxin. It is known that high concentrations of the digitalis glycosides have a central vasomotor effect(17) which implies stimulation of sympathetics and release of epinephrine. Bloom and Russell(14) show that epinephrine increases the total and labile glycogen in the heart. However, their results do not indicate an increase in bound glycogen which does occur with toxic doses of digoxin.

Summary. Digoxin in a dose of 0.2 mg/kg caused either no change or a slight increase in the cardiac glycogen fractions when administered to rats of 30 days of age or less, but caused a decrease in all fractions of cardiac glycogen when given to rats of 100 and 360 days of age. At 630 days of age, digoxin caused a fall in the trichloroacetic acid soluble fraction and an increase in the acid insoluble fraction with no change in total cardiac glycogen. When administered to 100- and 360-day-old rats toxic doses of digoxin increased all fractions of cardiac glycogen except the acid soluble fraction of the 100-day-old rats which showed no change.

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Human Nasal Cells in Continuous Culture.* I. Establishment of Two Lines of Epithelial-like Cells. (22638)

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The widespread application of tissue culture techniques to the study of the behavior of both malignant(1-3) and normal(4,5) human cells, and the use of such cells for the propagation of viruses(6-8) has resulted in the establishment of a number of strains of epithelial or epithelial-like cells in continuous culture. The present report describes the establishment of 2 lines of such cells from normal nasal mucosa in the search for tissue capable of supporting the growth of common cold viruses.

Material and Methods. The cultivation procedures were similar to those used by Chang(4) and to those used in this laboratory for the growth of HeLa cells(9). Nasal mucosa obtained at the time of elective surgery was minced and embedded in plasma clots in 18 × 150 mm roller tubes. *Media.* Homologous serum obtained from each donor was used to make growth fluid, as follows: 35% human serum, 5% chick embryo extract, and 60% Hanks' balanced salt solution. Final processing of the chick embryo extract included freezing and thawing twice followed by centrifugation at 18,000 rpm for 30 minutes. It had been stored at –20°C for 5 months prior to use. Penicillin and streptomycin were added to the growth fluid to give concentrations of 100 units and 0.1 mg per ml, respectively. Later, the media consisted of 40% of pooled human serum and 60% Hanks' solution with 5 units of polymixin B plus penicillin and streptomycin. For subcultivation, 0.1% trypsin was initially used to make cell suspensions, and 0.1 ml of 1% soya bean trypsin inhibitor was added per ml of medium. Following the establishment of sheets of epithelial-like cells in stationary flasks and bottles, 0.5% versene was substituted for the trypsin(10), and the inhibitor omitted. To prepare the versene 2.5 g of ethylene diamine tetra acetic acid was added to 500 ml of NaCl, the solution alkalinized with 5% $NaHCO_3$, and autoclaved. *Measurements.* Certain characteristics of the cells were measured as follows: Size was determined using stained preparations and living sheets in 24-hour-old tubes; the dimension of 100 cells in the plane of the scale was measured with an ocular screw micrometer. Growth was measured by adding 25,000 cells to each of 30 tubes and counting the number of cells in 4 of these tubes at 24 hour intervals. Details of the comparison of effect of trypsin and versene on growth rates constitute part of another study(10).

Luxuriant outgrowth was obtained with tissues from 2 of 4 donors, a 17-year-old female (DMB) and a 32-year-old male (DHov), and the subsequent behavior of these outgrowths was quite similar.

Experimental. DMB strain (*Fig. 1*). The cilia on the mucosal explants continued to beat actively for 7 days, but no cilia were observed on the outgrowth of new cells that became visible in 4 days. Nearly all of the cells that appeared and spread over the surface of the tube resembled fibroblasts, although occasional round cells and flat cells were observed in the cultures. After 24 days, the sheet of cells was trypsinized and new roller tubes and some small stationary tubes were made. The small tubes were used for virus studies(11), and the large tubes subcultured after 8 days. Again, the cells of the sheets which formed resembled fibroblasts.

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ESTABLISHMENT OF DMB NASAL CELLS

Explant $\xrightarrow[\text{days}]{4}$ Outgrowth $\xrightarrow[9]{5}$ "Fibroblasts" $\xrightarrow[24]{15}$ Subculture 1

"Fibroblasts" $\xrightarrow[32]{8}$ Subculture 2 $\longrightarrow$ "Fibroblasts" $\xrightarrow[44]{12}$

Subculture 3 $\longrightarrow$ "Fibroblasts" $\xrightarrow[52]{8}$ "Epithelial" Island $\xrightarrow[66]{14}$

Subculture 4 $\longrightarrow$ "Epithelial" cells $\xrightarrow[73]{7}$ Subculture 5 $\longrightarrow$

"Epithelial" cells $\xrightarrow[81]{8}$ Subculture 6 $\xrightarrow[\text{days}]{5-7}$ Subcultures 7 to 24

FIG. 1.

Figs. 1 and 3. Number above the arrow indicates number of days between events; number below the arrow indicates cumulative number of days since tissue was explanted.

However, 8 days following the third subculture and 52 days from the date of the original explant (Fig. 1) an island of epithelial-like cells was noted among the fibroblasts (Fig. 2). The cells of this island filled the space between the fibroblasts in an additional 14 days, producing a macroscopically visible mass of tissue. The island was then dissected from

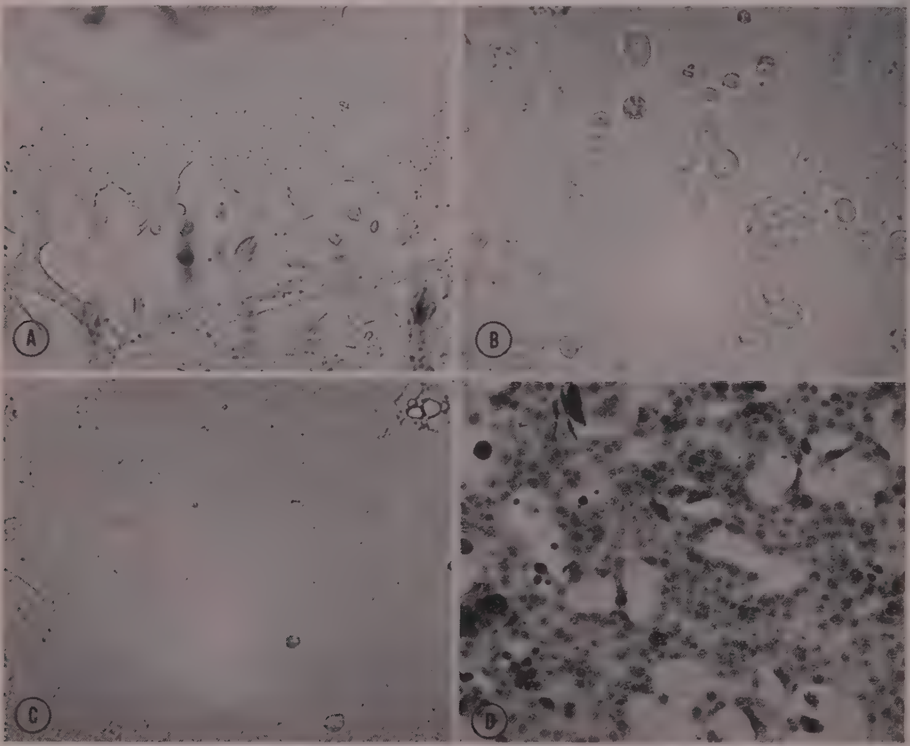

FIG. 2. A. Island of DMB cells which appeared 52 days after original explant. Photographed on 56th day. B. 24 hr culture of DMB cells; × 100. C. 4 day culture of DMB cells; × 100. D. DMB cells stained with EA_{50}; × 84.

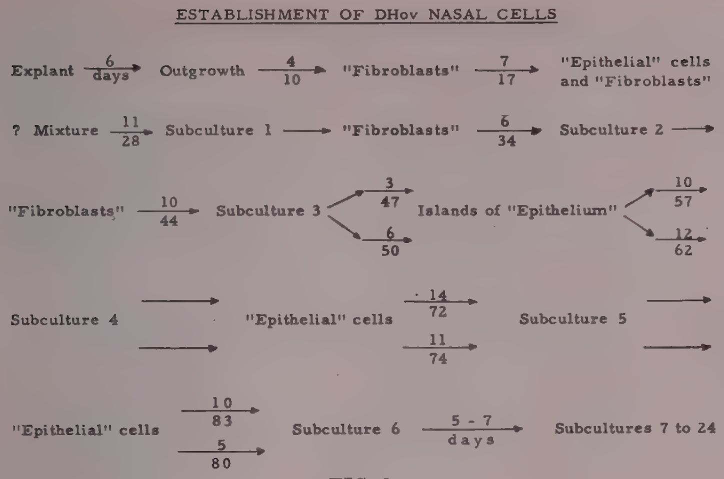

FIG. 3.

the sheet in the roller tubes, trypsinized, and the cells resuspended in growth fluid containing chick embryo extract. The sheet of epithelial-like cells which formed within a week was removed to make 2 roller tubes. Subsequently, cells from these tubes were transferred to T_{15} flasks, then to T_{60} flasks and small prescription bottles. The DMB strain has been maintained through more than 50 subcultures for over a year.

DHov strain (*Fig. 3*). The outgrowth from these explants also consisted predominantly of fibroblasts, but more round and polygonal cells were evident in among the elongated fibroblasts. In 1 of 6 original tubes, an area, not a discrete island, of the cellular sheet seemed to consist predominantly of epithelial cells. After 23 days, an attempt was made to remove this area, but the resulting subculture again consisted predominantly of fibroblasts. However, following a third subculture in a different series, islands of epithelial-like cells (Fig. 4) were noted in 2 different tubes 47 and 50 days from the beginning. These islands filled the gaps between the fibroblasts in 10 and 12 more days, and were then removed, trypsinized and resuspended in growth fluid containing embryo extract. Sheets of epithelial-like cells formed in 11 and 14 days. Cells of this strain have also been maintained through more than 50 subcultures for over a year.

Characteristics of cells. As cultivation of the DMB and DHov strains continued, the interval between subcultures could be shortened because of the rapid formation of complete sheets. Chick embryo extract was omitted from the feeding fluid, but rapid growth rates continued, and were similar to those for HeLa and Detroit 6 cells in the same medium (10). At 2 days, the number of cells had increased 2X; at 3 days, 4X; at 4 days, 6X; at 6 days, 10X. As with the cells of malignant origin, tubes ready for use in 24 hours can be produced by adding 60,000 cells per tube. Both strains are quite active metabolically, and require daily growth fluid changes. Mechanical disruption alone, satisfactory in the case of HeLa cells, did not produce good cell suspensions because of the tendency of the nasal cells, particularly the DMB strain, to clump. A 5 minute exposure to versene effectively removed the sheets and separated the cells(10). With versene, 98% of the cells observed in the hemocytometer were single cells. A maintenance fluid(12) used for HeLa cells has been satisfactory for the propagation of viruses in these nasal cells. The DMB strain holds better than the DHov strain and even better than HeLa cells. No truly satisfactory maintenance medium for DHov cells has been found. The titers of viruses, including poliomyelitis viruses, grown in HeLa cells are essentially the same in DMB, DHov, and

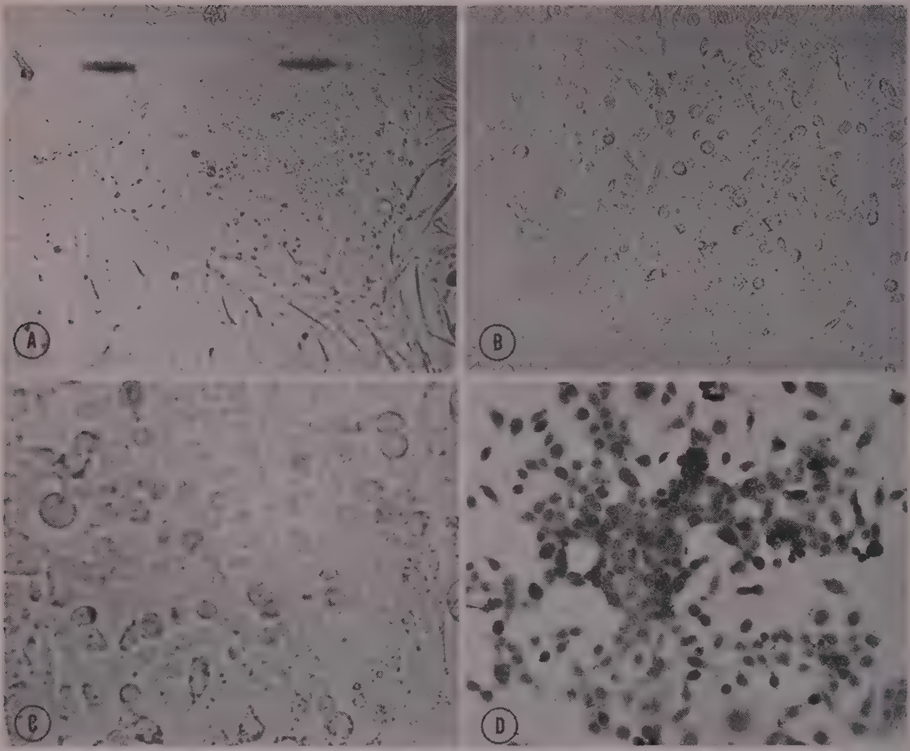

FIG. 4. A. Island of DHov cells which appeared 50 days after original explant. B. 24 hr cultures of DHov cells; × 50. C. 4 day culture of DHov cells; × 100. D. DHov cells stained with EA_{50}; × 84.

HeLa cells(11).

Although both strains were derived from nasal mucosa, the cells of the two lines differ morphologically. DMB cells (Fig. 2) are broad, flat, pavement-like cells. DHov cells (Fig. 4) are narrower, more elongated, angular and pointed, and a number of small round cells are persistently present. These differences in shape were readily apparent on inspection of the cultures although the cell sizes were similar. DMB cells measured as follows: Stained, 16 to 105 μ with average diameter of 36 μ; living, 16 to 88 μ with average diameter of 44 μ. DHov cells measured: stained, 13 to 101 μ with average diameter of 36 μ; living, 16 to 135 μ with average of 41 μ. As would be expected from the growth rates, many mitotic figures are seen in active cultures.

Cover slip preparations stained with EA_{50}, a polychrome stain used to bring out nuclear detail, were examined by Dr. James W. Reagan,[‡] a cytologist who has studied the morphology of malignant cells for a number of years. He reports that both DMB and DHov cells exhibit the following characteristics: cellular pleomorphism and gigantism, multinucleated cells, nuclear enlargement, variation in nuclear size and shape, altered chromatin patterns, large chromocenters, multiple nucleoli, macronucleoli, variation in nucleolar shapes, and abnormal mitoses. Dr. Reagan states that these are the *morphologic* features of malignant cells.

Cell suspensions therefore were injected into the anterior chamber of guinea pig eyes(13) and into the cheek pouches of cortisonized hamsters(14,15). No growth of tumor occurred in the guinea pig eyes, but since HeLa cells similarly inoculated failed to grow, these results are of no significance. The hamsters have been followed for 7 weeks without the appearance of tumor nodules. No human transplantation studies have been done.

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Discussion. The delayed appearances and subsequent rapid growth of epithelial-like cells in roller tube cultures of nasal mucosa have also been observed by Feller(16). The most logical explanation would seem to be that these cells were always present in the cultures, and eventually, for reasons unknown, were able to outgrow the fibroblasts and establish themselves. Subcultivation of the fibroblasts was continued in the same 40% human serum medium, but the fibroblasts of both lines failed rapidly after 60 days, and could not be maintained in continuous cultures. Chang(4) has discussed the possibility of chick embryo extract being the source of epithelial-like cells. The 2 cycles of freezing and thawing followed by ultra-centrifugation and storage make the presence of viable cells in the extract extremely unlikely. Further, as pointed out by Chang(4), the susceptibility of the cells to poliomyelitis virus indicated that they are of human and not of chick origin.

The emergence from normal tissue of cells with the cytologic characteristics of malignant cells has been reported in the past, and the implications of such an observation are many. Since normal mouse or rat fibroblasts can be transformed in tissue culture into malignant cells capable of producing sarcomas in the animals of origin(17-19), it is reasonable to assume that cells of human origin are capable of such transformation. Indeed, a recent report describes the transformation of normal human fibroblasts into histologically malignant tissue *in vitro*(20). It is believed that the DMB and DHov strains are epithelial, or certainly epithelial-like, cells, but at present the only evidence that these cells are malignant is their morphologic characteristics. The behavior of other epithelial cells(4,5) derived from normal tissue suggests that they may have similar properties. If malignant transformation of these and other strains can be demonstrated—and this will require the establishment of new criteria for malignancy—tissue culture will become an increasingly important tool for studying the origin and metabolism of cancer cells(21,22).

Summary. Two strains of epithelial-like cells derived from normal nasal mucosa have been maintained through more than 50 continuous subcultures for over a year. Both of these strains now have rapid growth rates, and exhibit the morphologic characteristics of malignant cells.

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Human Nasal Cells in Continuous Culture.* II. Virus Susceptibilities.
(22639)

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The cultivation of 2 strains of epithelial-like cells derived from normal nasal mucosa has been described(1). These cells, the DMB and DHov strains, can be distinguished morphologically, but share in common the capacity for rapid growth on glass and the presence of cytologic features characteristic of malignant cells(1). The present communication reports the susceptibility of these cells to a selected group of viruses.

Methods. The medium used for propagation of the cells consisted of 40% pooled human serum and 60% Hanks' balanced salt solution, the same feeding fluid used for the HeLa cells(2) with which the DMB and DHov strains were compared. Tubes for virus titrations were used 24 hours after inoculation of approximately 60,000 cells in 0.6 ml of media. Bottles for virus growth were used 4 to 5 days after the introduction of 10 ml of fluid containing 50,000 cells per ml. At the time of virus inoculation, these bottles contained approximately 2.5 × 10^6 cells. The maintenance fluid(3) contained 7.5% chicken serum, 17.5% tryptose phosphate broth and 75% Scherer's maintenance solution(4).

Viruses. The viruses employed were: 1) Adenoviruses types 1 through 7; type 4 was obtained from Dr. M. R. Hilleman, type 7 from Dr. T. O. Berge, and all other types from Dr. R. J. Huebner. 2) Poliomyelitis types 1, 2, and 3. 3) Coxsackie, Group B, type 3, originally isolated in HeLa cells(5). These agents had all been passed many times in HeLa cells. 4) Herpes simplex. 5) ECHO types 7 and 8, and 6) "chimp rhinitis", obtained from Dr. A. B. Sabin. 7) "CA" virus, Greer strain, obtained from Dr. R. M. Chanock(6). The ECHO and "CA" agents, originally isolated in monkey kidney cells, were passed two or three times in HeLa cells to provide the inocula used. 8) Measles (Enders' Edmonston strain) obtained from Dr. F. C. Robbins after passage in amnion cells, and passed once in HeLa cells. 9) Influenza A viruses (FW 1-53 and A/Clev/2/56) and influenza B viruses (Lee and GL/1/54). 10) Mumps virus (Habel strain). 11) Newcastle Disease Virus (4F and Hickham strains). These were all egg passage strains, and allantoic fluids were used as inocula for 18 × 150 mm roller tubes.

TABLE I. Comparative Virus Titrations* in HeLa Cells and 2 Lines of Nasal "Epithelial" Cells.

Virus		Cell line		
		HeLa	DMB	DHov
Adenovirus	Type 1	5.25	4.25	4.75
	2	4.75	4.0	4.0
	3	4.0	3.75	3.25
	4	3.75	3.5	3.0
	5	6.5	4.75	5.0
	6	6.25	4.5	4.25
	7	3.75	3.75	3.75
Poliomyelitis	Type 1	7.0	7.0	7.0
	2	6.3	6.0	5.7
	3	5.7	6.0	6.0
Coxsackie	Group B, 3	4.0	4.5	5.0
ECHO	Type 7	5.0	4.5	5.5
	8	4.5	4.5	4.5
Herpes simplex		3.25	3.75	2.25
"CA" Greer		4.25	4.0	3.75
Chimp "rhinitis"		3.75	3.75	3.75
Measles		3.0	2.5	2.75

* TCD_{50}/ml (log).

Titrations. Dilutions (1 : 3.2 or $10^{-0.5}$) of inocula were made in Hanks' balanced salt solution, and 0.1 ml of each dilution was added simultaneously to 0.9 ml of maintenance fluid in 2 tubes of HeLa, DMB, and

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† Markle Scholar in Medical Science.

TABLE II. Growth of Viruses in 2 Strains of Nasal "Epithelial" Cells.

	Cell line—TCD$_{50}$/ml (log)			
	DMB		DHov	
Virus	Inoculum*	Harvest	Inoculum*	Harvest
Adenovirus Type 2	1.5	3.75	1.5	3.5
4	1.7	3.5	1.7	3.0
7	1.0	2.75	1.0	2.75
Poliomyelitis Type 1	4.0	6.5	4.0	7.25
Coxsackie Group B, 3	3.3	5.25	3.3	5.0
ECHO Type 7	3.0	4.75	2.25	3.75
Herpes simplex	1.25	3.25	1.25	4.0
Chimp "rhinitis"	1.0	1.5	1.0	3.5

* Final concentration estimated from dilution in bottle.

DHov cells to give the comparisons shown in Table I. The tubes were inspected daily for the occurrence of degeneration, and alkalinized with NaHCO$_3$ if necessary. For the ECHO, "CA", and "chimp rhinitis" viruses end points were determined after 9 days; for the other viruses, after 6 days. With the exception of measles virus, end points were measured by the involvement of 50% or more of the cells. In the higher dilutions, measles virus produced plaques, but general involvement of the sheet of cells did not occur. Titer of measles virus is based on the highest dilution producing plaques.

Growth of virus. Multiplication of certain of the agents which produced definite cytopathogenic effects was assayed by harvesting the bottles after the appearance of maximum cytopathogenic effects. The cells were disrupted in the maintenance fluid by freezing and thawing 6 times. The supernatant was then titered in HeLa cells, and often in DMB and DHov cells as well. Each inoculum had previously been titrated in HeLa cells, and the amount of virus initially present in the bottles was calculated from the dilution of the inoculum (Table II).

Results. Fibroblasts predominated in the initial outgrowths from the explants that were the origin of both strains of nasal cells(1). With the first subcultures of these outgrowths, small tubes were made and the cells which grew, still seemingly all fibroblasts, were infected with type 4 adenovirus. Virus titering $10^{3.5}$ per ml was added in 0.1 ml amounts to 0.9 ml of maintenance fluid in DHov tubes. The maintenance fluid was completely replaced on the first and second days. Round cells slowly developed, and this time acidity was corrected by NaHCO$_3$. The titer of the material harvested on the seventh day was $10^{2.5}$ TCD$_{50}$ per ml, indicating that at least all of the virus added to the tubes had been recovered. This harvest was then added in 0.1 ml amounts to tubes containing DMB fibroblasts. This amount of virus produces degeneration of HeLa cells (and of DMB and DHov epithelial-like cells) in 24 hours. The development of cytopathogenic changes in the fibroblasts was slow (Fig. 1), reaching its maximum in 6 days. The maintenance fluid was replaced once during this time. The titer of the harvest was $10^{3.5}$ TCD$_{50}$ per ml, indicating a 10 to 100 fold increase in amount of virus. Thus, it was possible to demonstrate growth of an adenovirus in fibroblasts before the epithelial-like cells derived from the same explants of nasal mucosa had been established. Although its evolution was much slower, the cellular rounding and clumping that occurred (Fig. 1, D) was very similar to that noted with HeLa cells and the DMB and DHov epithelial-like strains. The fibroblasts could not be maintained in continuous culture, and further virus studies with these cells were not possible. All subsequent results were obtained with the epithelial-like cells.

Table I lists those agents which induced specific degeneration of DMB and DHov cells, and compares the titers obtained with these 2 strains with those measured with HeLa cells. These comparisons indicate that the sensitivities of these cells of nasal origin are virtually the same as those of HeLa cells. The DHov cells are least satisfactory because many round cells appear in the several maintenance fluids tried. There is no evidence as yet that this change is due to the presence of a latent virus in the DHov strain. Representative types of the various agents were used to determine whether cellular degeneration was accompanied by viral multiplication (Table II). As suggested by the comparative titrations, the yield of virus was similar to that obtained with HeLa cells. The change induced in DMB

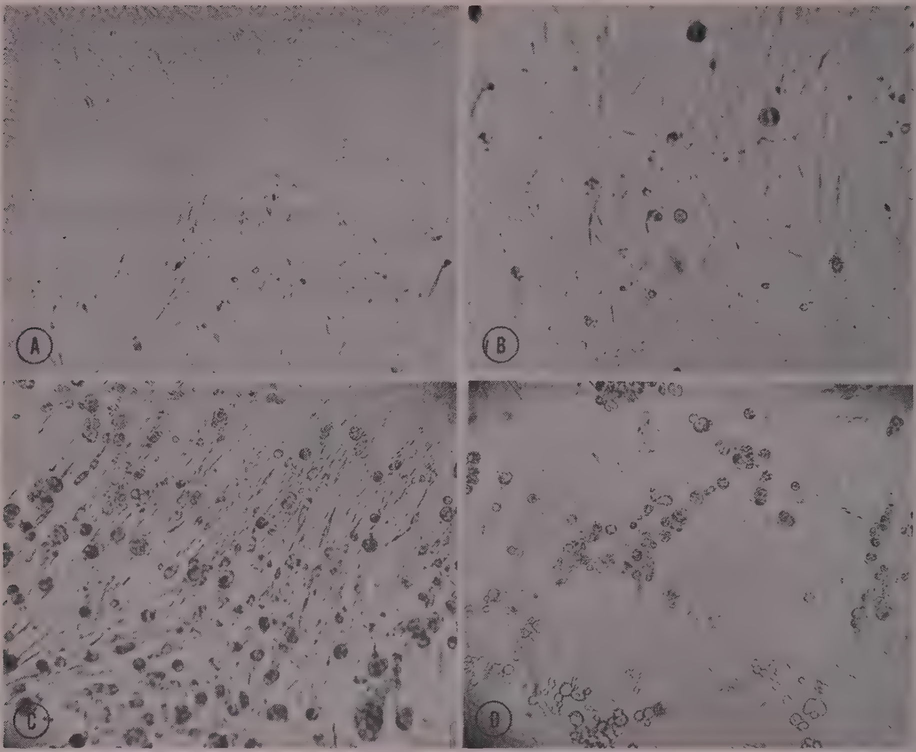

FIG. 1. Growth of type 4 adenovirus in early cultures of DMB cells when fibroblast was predominant cell type. A. Uninfected culture. B. 48 hr after addition of about 300 TCD_{50} of virus. C. 96 hr after infection. D. 144 hr after infection.

and Dhov cells by adenoviruses, polioviruses, Coxsackie, and herpes viruses were similar to those seen with HeLa cells(7). This was also true of the other agents, but the changes have not been so generally described. The ECHO viruses tested produced small round cells, most of which eventually fell off the glass. A similar effect was induced by the "chimp rhinitis" agent. The evolution of the change was slow, maximum titers being obtained in 8 to 9 days. The rounded cells tended to be "ragged" and were not so spherical or so dense as poliovirus infected cells.

"CA" virus also produced its maximum effect in 9 days, and the unique changes that developed beginning on the fourth day might easily have been mistaken for poor cell maintenance. There was a progression of changes which resembled the accumulation of debris plus the production of round cells. Certain areas of degeneration resembled the "spongy" effect noted on the kidney cells by Chanock (6). In other areas fragmented sheets of cells were plastered with debris which seemed to cling like iron filings on a magnet.

Measles virus produced syncytial giant cells, but the cytoplasmic vacuoles were less obvious than described for monkey kidney cells(8). In addition, collections of material resembling steel wool, reminiscent of the effect of "CA" virus, appeared. The $10^{-0.5}$ and $10^{-1.0}$ dilutions of inoculum with a titer of $10^{3.5}$ per ml led to degeneration of nearly all cells in 4 and 5 days, respectively. With the higher dilutions, as noted above, local areas developed, but generalized involvement of the sheet of cells did not occur.

Influenza and mumps viruses did not induce cytopathogenic effects. Newcastle disease virus caused death of cells on initial passage; this was interpreted as toxic degeneration since it did not appear on subsequent passages.

Nasal secretions or washings collected from 6 adult donors with illnesses resembling the common cold have been passed 4 to 5 times in both DMB and DHov cells. A type 4 adenovirus was isolated from an individual who had just started to work with this agent in the laboratory, and who had an increase in titer of type 4 neutralizing antibody. Cytopathogenic agents have not been isolated from the other specimens.

Summary. (1) Two strains of epithelial-like cells derived from nasal mucosa have been found to be susceptible to a number of viruses. The fibroblasts which predominated in the early passages of these cell lines were susceptible, although degeneration occurred at a slower rate, to type 4 adenovirus. (2) Cytopathogenic effects were produced by 7 adenoviruses, 3 polioviruses, 2 ECHO viruses, a Group B Coxsackie virus, herpes simplex, measles, "CA", and "chimp rhinitis" viruses. Titers obtained in the nasal epithelial-like cells are similar to those measured with HeLa cells. Multiplication of certain of these agents was confirmed by titration of passage material. (3) Influenza and mumps viruses and nasal secretions from individuals with common colds were not cytopathogenic.

The technical assistance of Miss Virginia Colville and Mrs. Juanita Ruffier is gratefully acknowledged.

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Lysozyme: Its Characteristics in Human Parotid and Submaxillo-Lingual Saliva. (22640)

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Lysozyme activity in human saliva was reported by Fleming along with his discovery of the enzyme in 1922(1). It has been shown that human parotid saliva exhibits greater lysozyme activity than the corresponding whole saliva(2). This has led to the conclusion that the parotid glands are the major contributors to the total salivary lysozyme titer, if not the sole contributors. We were able to corroborate this finding employing turbidimetric assay methods(3). However, the lower lysozyme activity in whole saliva was due, in part, to increases in optical density resulting from differing concentrations of salivary sediment in the reaction systems. In this study, to avoid this source of error, salivas were collected by methods other than expectoration. Lysozyme activity was determined in sediment-free individual salivary gland secretions. As a result, lysozyme titers in the mixed secretions of the submaxillary and sublingual glands were found to be considerably higher than those in the corresponding parotid salivas. Data are presented regarding some of the characteristics of parotid and submaxillo-lingual* salivary lysozyme. Special emphasis is placed upon the interaction of parotid and SM-L saliva and the effect of this interaction upon the resulting total salivary lysozyme titer.

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* Henceforth submaxillo-lingual will be abbreviated as SM-L.

Methods. (a) *Lysozyme quantitation*: The turbidimetric lysozyme assay method of Smolelis and Hartsell(4) was adapted for our studies of salivary lysozyme. The optical density of a suspension of *Micrococcus lysodeikticus* cells, subsequent to exposure to an unknown saliva sample, was related to a previously prepared standard curve employing chromatographically homogeneous crystalline egg white lysozyme[†] as the primary standard. Accordingly, salivary lysozyme activity could be expressed as micrograms of enzyme per ml of saliva. These quantitative values, however, were not absolute, but represented egg white lysozyme equivalents. (b) *Saliva collections*: Parotid saliva was collected with the device and according to the method described by Curby(5). SM-L saliva was considered that secretion drawn from the floor of the mouth by gentle negative pressure into a sterile filtration tube after placing Curby caps over the right and left Stenson duct orifices. This method of collection yielded a clear mucoid fluid considered to be an expression of the submaxillary and sublingual glands. Parotid saliva, when collected singly, was stimulated by chewing sweetened gum. SM-L and parotid salivas, when obtained simultaneously, were stimulated by applying a small quantity of 3% acetic acid to the tip of the tongue.

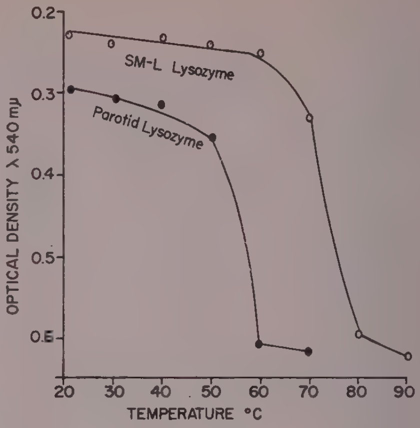

FIG. 1. Effect of heating on salivary lysozyme activity. Saliva samples were held at each temperature for 45 min. before activity tests.

Results. (a) *Parotid saliva*: Parotid saliva lysozyme tests were made at dilutions of 1:10 in the reaction system. The mean lysozyme titer in the secretions of the right glands of 100 normal male Naval personnel,[‡] age 17 to 26, was 23.1 ± 14.3[§] μg/ml. Values ranged from 4.5 to 80.0 μg/ml. The mean lysozyme titer in the mixed right and left gland secretions, collected simultaneously with SM-L saliva, in 30 individuals, was 15.0 ± 9.2 μg/ml. Values ranged from 5.0 to 42.0 μg/ml. The pH optimum for parotid lysozyme activity was 6.2 (Sørensons M/15 phosphate buffer). Total loss of enzyme activity was encountered upon heating parotid saliva at 60°C for 45 minutes (Fig. 1). (b) *Submaxillo-lingual saliva*: SM-L saliva lysozyme tests were made at dilutions of 1:20. The mean lysozyme titer for 30 individuals was

TABLE I. Lysozyme Titers in Parotid, Submaxillo-Lingual (SM-L) and Equimixtures of Both Salivas.

Lysozyme (μg/ml)				
		Parotid and (SM-L)		Difference
Parotid	(SM-L)	Expected	Observed	
5.0	38.0	43.0	42.5	− .5
5.0	38.0	43.0	41.5	− 1.5
5.5	47.0	52.5	50.0	− 2.5
5.5	90.0	95.5	88.0	− 7.5
5.7	62.0	67.7	64.0	− 3.7
6.0	9.0	15.0	20.5	+ 5.5
6.4	64.0	70.4	50.0	−20.4
8.0	76.0	84.0	76.0	− 8.0
8.5	90.0	98.5	87.0	−11.5
9.0	50.0	59.0	53.0	− 6.0
9.5	68.0	77.5	76.0	− 1.5
9.6	88.0	97.6	88.0	− 9.6
10.5	74.0	84.5	66.0	−18.5
10.5	148.0	158.5	130.0	−28.5
11.0	82.0	93.0	82.0	−11.0
11.0	87.0	98.0	87.0	−11.0
12.0	92.0	105.0	90.0	−15.0
13.0	50.0	63.0	65.0	+ 2.0
16.6	37.0	53.6	64.0	+ 9.4
18.7	82.0	100.7	76.0	−24.7
20.0	70.0	90.0	72.0	−18.0
20.0	69.0	89.0	73.0	−16.0
20.5	65.0	85.5	74.0	−11.5
21.0	70.0	91.0	70.0	−21.0
23.2	94.0	111.2	94.0	−23.2
25.0	148.0	173.0	148.0	−25.0
25.0	80.0	105.0	80.0	−25.0
27.0	94.0	121.0	97.0	−24.0
34.0	38.0	72.0	44.5	−27.5
42.0	104.5	146.0	104.0	−42.0

[†] Difco Laboratories, Detroit, Mich.

[‡] Henceforth all cases fall into this classification.

[§] Group mean with standard deviation.

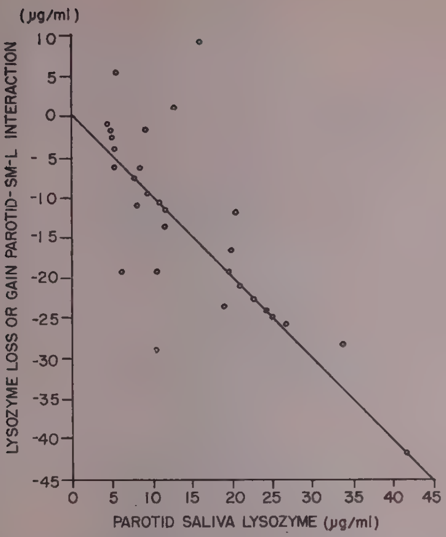

FIG. 2. Relationship of parotid lysozyme titers in each of 30 individuals to loss or gain in lysozyme activity in equimixtures of their parotid and submaxillo-lingual saliva.

73.4 ± 29.3 µg/ml. Values ranged from 9.0 to 148.0 µg/ml. The pH optimum was 6.2. Fig. 1 shows that total loss of enzyme activity occurred upon heating SM-L saliva at 90°C for 45 minutes. This remarkable thermostability of the SM-L enzyme is undoubtedly due to high concentrations of protein in the secretion. (c) *SM-L-parotid saliva interaction*: Lysozyme quantitations were made in equimixtures of SM-L and parotid salivas (1:20 dilutions) of the 30 individuals previously mentioned. The results of these tests appear in Table I. The observed lysozyme titers of the SM-L-parotid saliva interaction were, in all but 3 cases, less than the calculated expected titers. A plot of each parotid lysozyme value *vs.* the corresponding difference between the observed and expected value of the SM-L-parotid saliva interaction revealed a positive correlation (Fig. 2), suggesting existence of a component in the SM-L saliva capable of selectively inhibiting parotid saliva lysozyme activity.

Kaiser(6) has shown lysozyme inhibitory powers in electronegative chain molecules containing sulfur, *e.g.*, heparin, sulphonated hyaluronic acid, and sulphonated carboxy cellulose. Mucoitin sulfate, a monosulfuric acid ester of hyaluronic acid, occurs as the prosthetic group associated with gastric and salivary mucin(7). Accordingly, the "mucopolysaccharide"[∥] component of 4 ml of SM-L saliva was isolated by a modification of the method described by Winzler, Devor, Mehl and Smyth(8). By dry weight, 5.8 mg of the material was obtained. This material was suspended in M/15 phosphate buffer, pH 6.2, and added to parotid lysozyme reaction systems in concentrations ranging from 0.1 to 1.0 mg/ml of parotid saliva. The SM-L "mucopolysaccharide" inhibited parotid lysozyme activity in a linear fashion up to 0.4 mg/ml (Fig. 3). At the higher concentrations a consistent reduction of approximately 75% in parotid lysozyme activity occurred. The SM-L "mucopolysaccharide" exhibited no lysozyme activity *per se.*

Discussion. The difference in the group means of the parotid saliva lysozyme titers found in the 100 right gland tests and in the 30 mixed right and left gland tests was significant. The values in each group, and particularly in the right gland group, were abnormally distributed so that the means were

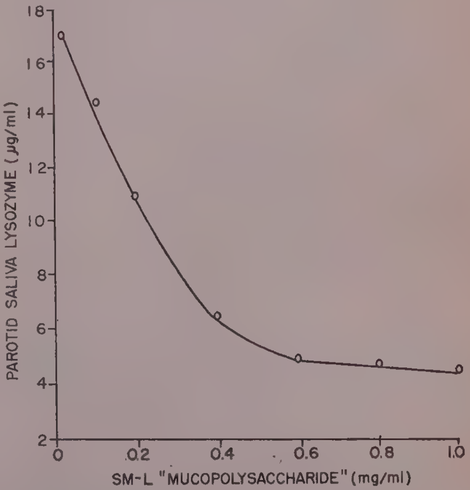

FIG. 3. The inhibitory effect of isolated "mucopolysaccharide" in submaxillo-lingual saliva on parotid saliva lysozyme activity.

[∥] Mucopolysaccharide is placed in quotations since absolute identification and purity have not been determined.

not representative of the groups. Modal values, as determined by the Δ method(9), were essentially the same; right gland group 10.0 μg/ml and mixed right and left gland group 9.0 μg/ml. The apparent variability in parotid saliva lysozyme titers encountered in this study could be accounted for by the different methods of gland stimulation and/or by the ability of the parotid gland to produce lysozyme in varying amounts according to the particular nervous stimulation present. In the course of our work with SM-L-parotid saliva interaction, it was observed that heat inactivated SM-L saliva (90°C for 45 minutes) lost its ability to inhibit parotid saliva lysozyme activity. This finding would tend to discount an inhibition of a non-competitive nature. On the other hand, to call the SM-L "mucopolysaccharide" inhibition of the parotid lysozyme selectively competitive would imply greater specificity for the inhibitor than for the substrate in the *Micrococcus lysodeikticus* cells and this would be invalid without knowing more of the direct mechanism of the inhibitory reaction. Lysozyme activity in SM-L saliva does not appear to be inhibited by the "mucopolysaccharide" component present therein. In this regard, Lindley(10) draws attention to the fact that 2 enzymes, both requiring the same group for activity may nevertheless show marked differences in behavior toward the same inhibitor. It is probable that such a system exists in the SM-L-parotid saliva interaction. Recently, lysozyme has been implicated as a determinant in the integrity of mucous membranes; especially those of the alimentary canal(11). Meyer, Prudden, Lehman, and Steinberg found high titers of gastric and intestinal lysozyme occurring concomitantly with (and many times preceding) ulcerative gastrointestinal disease. Therefore, it is tempting to speculate upon the role of salivary lysozyme in the etiology and course of ulcerative gingival disease. A possibility exists that a diminished quality or quantity of salivary mucoid and/or an abnormally high parotid lysozyme titer could play an important part in the etiology of ulcerative gingival disease.

Summary. 1. Lysozyme titers in secretions of the submaxillary and sublingual salivary glands were found to be substantially higher than those in the parotid gland secretions. 2. Parotid saliva lysozyme titers were variable. Means of the groups tested were considered equivocal. 3. A component of submaxillo-lingual saliva, isolated as the "mucopolysaccharide" group, exerted marked inhibition upon lysozyme activity in parotid saliva. This inhibition was probably competitive in nature.

The opinions or assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Department of the Navy, or the Naval Service at large.

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Renal Clearance of Fluoride.* (22641)

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The available evidence as reviewed by Hodge and Smith(1) indicates that rapid urinary excretion of fluoride is one of two major means by which the body prevents the accumulation of sufficient fluoride ion to reach toxic levels. Approximately half to two-thirds of the fluoride ingested is removed in this manner; the balance is deposited in the skeleton.

It would be unnecessary to assume that a special excretory mechanism were involved if the renal clearance of fluoride could account for the prompt appearance in the urine of a sizeable fraction of a small oral dose(1) and for the prompt disappearance of blood-borne fluoride after intraperitoneal(2) or intravenous(3) doses. Indications that this hypothesis is tenable were found in data from a fluoride balance experiment in rabbits(4); the tubular resorption of fluoride apparently was less efficient than that of sodium, chloride or water.

In this report renal clearance of fluoride by the female dog has been measured under a variety of conditions. Even at normal blood fluoride concentrations, in the microgram per 100 ml range, fluoride exhibited a rapid clearance, exceeding the simultaneously measured water or chloride clearances.

Methods. Renal clearance experiments were performed on 4 female mongrel dogs weighing 9.7 to 11.8 kg. These dogs were maintained on a stock fox chow ration (containing ca. 13 ppm F) and drank tap water containing 1.0 ppm F (artificially fluoridated). Since the experiments were designed to measure clearance and not balance, the exact fluoride intake was not determined.

During clearance periods the animals were unanesthetized and loosely restrained in the supine position. Urine was collected through Foley inflatable catheters; the collection was terminated by 2 bladder washings with distilled water and one with air. Food was withdrawn 24 hours prior to each experiment, but tap water was allowed *ad libitum* unless indicated. With normal fluoride intake and excretion rates, *i.e.*, no intravenous fluoride given, the clearance periods were 20 to 60 minutes. The periods were shortened to 10 minutes when fluoride was injected by vein.

Fifteen ml blood samples were withdrawn into heparin from a jugular vein at the midpoint of each clearance period. When fluoride was not injected, the blood contained so little fluoride that the plasma from 3 samples was pooled to provide an adequate sample for accurate analysis. Presumably this value reliably estimated the control blood fluoride concentration during the 3 hour test period.

Glomerular filtration rates were estimated by the clearance of endogenous creatinine. Hare's method(5), slightly modified, was employed. Flame photometry was used to measure sodium(6). The Fiske-Subbarow method was used for phosphate analyses(7). Fluoride in plasma and urine was determined by the methods of Smith and Gardner(8,9) and chloride by titration with mercuric nitrate (10).

Low rates of urine flow were obtained by withdrawal of water for 27 hours preceding tests. Diuresis was induced a) by administering tap or distilled water via stomach tube, and b) by administering hypertonic solutions of mannitol or salts. Intravenous injections and infusions were made into the jugular vein opposite that used for blood withdrawal.

Results. Ultrafilterability of Fluoride in Dog Plasma. As an indication of whether the fluoride in dog plasma is bound to non-

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* This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N. Y.

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filterable proteins, the fluoride concentration in an ultrafiltrate of plasma was measured. Samples of normal plasma and of plasma containing 1.4, 6.6 and 10.5 μg/ml added fluoride were ultrafiltered through seamless visking casing using the centrifuge type apparatus of Toribara(11). There was no difference between the fluoride concentrations in the ultrafiltrate and in the original plasma. Since fluoride moved through the visking membrane at the same rate as did water, the total plasma concentration can be used in calculating the renal clearance of fluoride.

TABLE I. Normal Fluoride Clearance in the Dog.

Urine flow	GFR	C_{Cl^-}	C_{F^-}	Plasma F, μg/100 ml*
		Water *ad libitum*		
.10	32.1	.20	.73	60
.10	33.1	.12	.77	60
.10	35.0	.20	2.7	29
.12	29.0	.16	1.5	42
.13	32.0	.10	1.2	46
.15	35.6	.42	1.4	60
.17	33.5	.22	1.4	46
.20	37.6		1.8	28
.27	40.5		2.0	28
.33	39.1		2.0	28
.40	34.3	.29	1.6	46
.65	29.6	.15	1.2	61
.67	43.5	.41	4.0	34
Avg	35.0	.33	1.7	
		Adm. distilled water		
.23	34.7	.29	2.6	29
.40	28.3		2.0	(36)
.60	28.5	.05	2.5	(36)
.83	30.0	.04	2.3	(36)
1.07	42.0	.18	2.8	34
1.55	44.5	.30	4.1	34
1.70	33.4	.25	2.5	29
Avg	34.5	.17	2.9	
		Adm. tap water		
.12	29.6	.12	2.2	42
1.47	33.5	.12	4.5	42
1.84	29.2	.12	3.5	42
2.45	39.4	.11	2.1	61
2.92	34.3	.09	1.9	61
Avg	33.2	.11	2.8	
		Water withdrawn 27 hr		
.05	30.7	.15	4.3	15
.07	31.4	.12	3.2	12
.07	33.0	.22	4.1	15
.08	36.5	.26	7.2	15
.08	35.2	.16	2.9	12
.17	36.1	.14	4.1	12
Avg	33.8	.17	4.3	
Overall avg	34.4	.18	2.7	36
Range		.04-.42	.73-7.2	12-61

* Values within parentheses taken from pooled plasma samples.

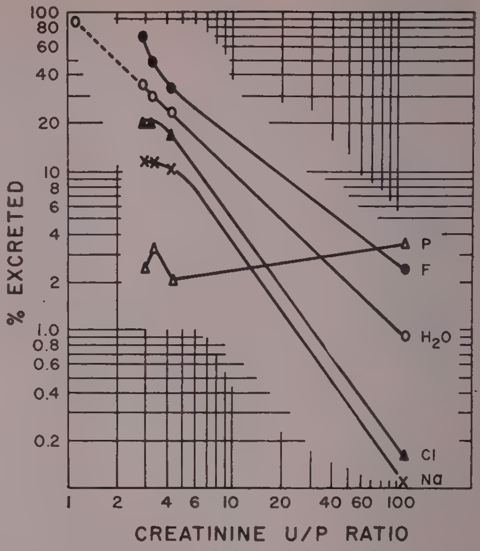

FIG. 1. Percentage of filtered ion and water excreted at various creatinine U:P ratios during hypertonic mannitol infusion.

In one instance where fluoride was added to plasma, chloride also was measured in the plasma and in the ultrafiltrate; no difference was found.

Normal fluoride clearance. A series of normal fluoride clearance measurements was made. The urine flow varied from 0.05 to nearly 3 ml/min. (Table I). Collection periods each were 60 minutes. In the control group without special treatment (water *ad libitum*) fluoride clearances averaged 1.7 ml/min., with an average fluoride : chloride clearance ratio of 7 and fluoride : creatinine clearance ratio of 0.048. Thus, in this group, 4.8% of the filtered fluoride was excreted against a simultaneous chloride value of 95%. At elevated or reduced rates of urine flow established by the administration of distilled water or tap water or by water withdrawal, fluoride clearances increased to 2.9, 2.8 and 4.3 ml/min., respectively. Chloride clearances, on the other hand decreased to 0.17, 0.11, and 0.17 ml/min. Therefore in these 3 control groups, average fluoride : chloride clearance ratios (27, 25, 25) were considerably higher than in the water *ad libitum* group, as were also the average fluoride : creatinine clearance

TABLE II. Effect of Hypertonic Salt Infusion on Fluoride Clearance.

	Time, min.	Urine flow	GFR	C_{Cl^-}	C_{F^-}	C_{F^-}/C_{Cl^-}	C_{F^-}/GFR
Hypertonic NaCl	0						
	20	.01	28.5	.062	3.9	63	.14
	27	Began infusion of 5% NaCl at 8.5 ml/min. T.V. = 255 ml					
	34	Washed bladder. Start of exp. periods					
	44	1.5	34.8	3.08	6.7	2.2	.19
	54	5.0	33.7	7.8	4.7	.6	.14
	64	8.8	42.8	13.1	9.8	.75	.23
Hypertonic NaCl c fluoride	0	Began infusion of 5% NaCl containing 100 mg F/ml at 10.0 ml/min. T.V. = 350 ml					
	15	Start of exp. periods					
	25	4.5	45.3	7.0	21.8	3.1	.48
	35	6.7	49.2	—	28.0	—	.57
	45	6.1	44.6	8.2	24.5	3.0	.55
Hypertonic $NaNO_3$	0	Adm. 300 ml distilled water by stomach tube					
	30	Start of control period					
	50	.09	48.5	.04	2.5	62.5	.05
	51	Began infusion of 2.6% $NaNO_3$ at 5 ml/min. T.V. = 280 ml					
	65	Washed bladder. Start of exp. periods					
	85	1.7	46.0	2.4	2.8	1.17	.06
	105	5.4	48.3	6.7	3.2	.48	.07
Hypertonic Na_2SO_4	0	Adm. 350 ml distilled water by stomach tube					
	10	Start of control period					
	30	.15	26.1	.04	7.7	192	.30
	35	Began infusion of 2.8% Na_2SO_4 at 7 ml/min. T.V. = 425 ml					
	50	Washed bladder. Start of exp. periods					
	70	7.3	31.0	.39	7.1	18.2	.23
	90	7.5	32.2	1.49	4.9	3.3	.15

ratios (0.081, 0.087, 0.126). Plasma concentrations of fluoride averaged 36 μg/100 ml with a range of 12-61. The blood level of fluoride was lower after water was withdrawn for 27 hours. Water was provided *ad libitum* for one control period in each of the water diuresis experiments; these data are listed in the "water *ad libitum*" group and account for the greater number of these periods.

Effect of Mannitol Diuresis on Fluoride Excretion. The experimental technique was as follows: 250 ml of a 25% mannitol solution containing 125 μg F^-/100 ml was infused i.v. at a rate of 6-7 ml/min., and urine was collected for three 10 min. periods following a 12 min. equilibration. Infusion of hypertonic mannitol evoked a considerable diuresis, with maximal urine production approximating one third of the glomerular filtration rate. Under these conditions the composition of the urine approaches that of the fluid entering the distal tubules(12). A representative mannitol diuresis experiment is plotted in Fig. 1. The percentage excretion of filtered substance has been plotted on a log-log scale *vs.* the creatinine U : P ratio. In these experiments the minimal creatinine U : P ratio was 2.9 and the maximal ratio was over 100. Phosphate excretion was practically independent of creatinine U : P ratios; 2 to 4% of the phosphate was excreted in each test. As the creatinine U : P ratio approached 1.0, the percentage of sodium and of chloride which escaped tubular reabsorption was less than that for water. This approach has led to presumptive evidence for the active proximal tubular reabsorption of sodium(12) and calcium(13). The fluoride excretion curve lies roughly parallel to that for water but above it. At all urine flow rates, fluoride was excreted with a clearance much greater than that of water, sodium, chloride or phosphate.

Effect of Salt Diuresis on Fluoride Excretion. Osmotic diuresis using hypertonic NaCl, $NaNO_3$, and Na_2SO_4 was employed to observe its effect on fluoride excretion. In addition

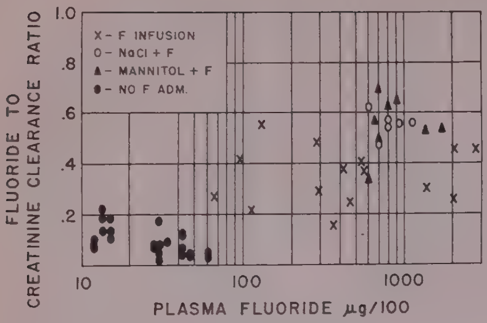

FIG. 2. Fluoride : creatinine clearance ratios at various fluoride plasma concentrations.

to increasing the urinary rate, the high plasma concentrations of such anions might be expected to interfere with or enhance fluoride clearance. The data are shown in Table II and Fig. 2. No significant increase of fluoride clearance above control levels followed the infusion of hypertonic NaNO$_3$ or Na$_2$SO$_4$ even though a large diuresis of water, sodium, chloride, and presumably infused anion occurred. Hypertonic NaCl did increase fluoride clearance appreciably, although the chloride clearance exceeded that of fluoride in the last 2 experimental periods. When a fluoride-containing hypertonic NaCl solution was infused, the fluoride clearance was actually greater than if only fluoride (NaF) had been used to raise the plasma fluoride (Table II). This tendency for a more rapid excretion of fluoride during NaCl diuresis was similar to that seen with mannitol diuresis.

Discussion. Renal clearance of fluoride at normal plasma levels in the dog, *viz.*, 12-61 μg F/100 ml (0.0065-0.033 mEq./l.) was rapid. For example, average normal excretion of filtered fluoride was 7.7% with extreme ranges of 2.3 and 13.9%. Few physiologically occurring anions are cleared by the kidney with such rapidity.

The fluoride clearance values (0.73-7.2 ml/min.) can be compared with available data on such anions as chloride, bromide, and phosphate. The normal chloride clearance is less than 1% of the glomerular filtration rate (averaging 0.5% in this study). Average clearance ratios of 0.6 for bromide : chloride (14) have been reported for the dog, a value much smaller than that for fluoride : chloride. However, an interdependence of bromide with chloride excretion was demonstrated, chloride loading increasing bromide : chloride excretion. In this respect these halogens, fluoride and bromide, are similar in that diuresis increased fluoride excretion. Nearly quantitative renal resorption of phosphate(14) occurs at normal plasma levels. The mechanism of its active resorption has a limited capacity and can be saturated, whereupon its clearance approaches that of creatinine. Osmotic diuresis should not affect the excretion of this ion. Fig. 1 shows that phosphate excretion did not vary with the creatinine U/P ratio. Fluoride, in contrast to chloride, bromide, and phosphate, demonstrated a high rate of renal clearance at the relatively low plasma concentrations attained upon fluoride administration. For example, the fluoride : creatinine clearance ratios, with only one exception, lay between 0.2 and 0.7 at plasma fluoride levels between 58-2883 μg/100 ml (Fig. 2). The highest fluoride : creatinine clearance ratios were obtained during mannitol diuresis (Fig. 2). Since fluoride clearance was always well below the creatinine clearance, there was no evidence in these experiments that tubular secretion occurred. A similar conclusion was drawn from estimations of the fluoride clearance in rabbits(4).

Summary. Fluoride in dog plasma was completely ultrafilterable through visking membranes when tested using the centrifuge type apparatus. In dogs with a normal water load, the average normal renal fluoride clearance was 2.7 ml/min.; the fluoride : chloride clearance ratio, 19; and the fluoride : creatinine clearance ratio, 0.077. Fluoride clearance was more rapid than that of simultaneously measured sodium or chloride clearances or urine flow. During mannitol diuresis, fluoride was cleared at a rate higher than that of sodium, chloride, or phosphate. Fluoride clearance, although greater than urinary flow, varied directly with the flow under mannitol osmotic diuresis. Intravenous infusion of hypertonic NaNO$_3$ or Na$_2$SO$_4$ solutions, creating a salt diuresis, did not affect fluoride excretion. Hypertonic NaCl infusion did increase the fluoride clearance. Although fluoride : creatinine clearance ratios were raised greatly by intravenous fluoride, the highest ratios were attained during fluoride infusion plus osmotic diuresis with hypertonic mannitol or NaCl. No evidence for a tubular secretion of fluoride was obtained.

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Rapid Diagnosis of Human Influenza Infection from Nasal Smears by Means of Fluorescein-Labeled Antibody.*† (22642)

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Laboratory diagnosis of influenzal infection is usually carried out by means of retrospective serological reactions or by isolation of the virus from throat washings. Attempts have been made to shorten the time for diagnosis. Hummeler *et al.*(1) used the complement fixation test with known serum and the soluble antigen from primary isolation of the virus in chick embryos. He reported that typing of the virus was successful in 48 to 72 hours after collection of specimens. Fazekas de St. Groth(2) found that the "inhibitory index" to influenza virus of human nasal mucus was significantly increased during the acute stage of infection, allowing an early presumptive diagnosis of influenzal infections.

In the study of influenzal infection in ferrets by means of fluorescein-labeled antibody, it was possible to make a rapid diagnosis of influenzal infection during the acute stage of illness by detecting specific viral antigen in the desquamated nasal epithelial cells and macrophages in the nasal smears(3). This paper reports the application of this method to human influenzal infection. The results indicate that, although the staining of nasal smears by fluorescein-labeled antibody is less sensitive than the standard hemagglutination-inhibition test for the diagnosis of influenzal infection, it is much quicker. A diagnosis can be made on the same day as the collection of the nasal washings.

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Materials and methods. Most of the patients were young male students admitted to the infirmaries of Harvard University, Cambridge, Mass. or Phillips Exeter Academy in Exeter, N. H. with a clinical diagnosis of influenza or acute upper respiratory infection. The patients at Harvard were examined by the author and the patients in Exeter were examined by Dr. James T. Heyl. On all patients, nasal washings and a sample of venous blood were collected during the first visit while they were at the infirmary. The convalescent blood samples were collected 2 to 3 weeks after they were discharged.

Preparation of nasal smears. Nasal washings were performed with the patient in a supine position, and the neck fully extended. With a 10 ml pipette, 5 ml of normal 0.85% saline was introduced into each nostril. (In a few cases, beef heart infusion broth was used instead of saline.) After instillation of the solution, the patient was allowed to sit up to let the saline drip out and the liquid was collected into a Petri dish. Usually about 4 to 5 ml of liquid was recovered from both nostrils. About one ml of the washings was put into a test tube, sealed and stored in a dry CO_2 box to be used for virus isolation. The remainder was transferred to a test tube and centrifuged at about 1,000 rpm for 5 minutes. The supernatant fluid was removed by a capillary pipette, leaving about 0.2 ml of liquid. The cellular sediment was then dispersed in this small volume of saline. A drop of the cell suspension was spread over an area of about 1 cm in diameter on a 3 x 1 inch glass slide. Four to 5 slides were made from each nasal washing. The slides were dried in air at room temperature and then kept in the refrigerator at 4°C. Slides may also be dried directly in the refrigerator. In a few patients, a small cotton swab wetted with saline was introduced into the upper nasal passage with the aid of a nasal speculum and a head mirror. The nasal turbinates were touched by the swab, and smears were made by rolling the swab on a glass slide.

Fluorescein-labeled antisera were prepared according to the method of Coons and Kaplan(4). The specific influenza sera against influenza A (Farrington and Fm_1 strains) and the Lee strain of influenza B viruses were prepared in rabbits as described previously(3). Before use as a histochemical stain on nasal smears, the fluorescent antibody solutions were absorbed twice with ferret liver powder and once with rabbit bone marrow powder. The latter absorption is for the removal of non-specific fluorescence in white blood cells, especially the granulocytes(5).

Fixation, staining and examination of slides. Nasal smears were fixed in acetone for 10 minutes and dried in a 37°C oven for 20 minutes. Usually 2 slides were fixed and dried in this way. For staining, one slide was treated with a drop of influenza A fluorescein-labeled antibody solution for 30 minutes at room temperature; the other slide was treated in a similar manner with a fluorescent influenza B antibody. Afterwards, the slides were washed in buffered saline (pH 7.0) for 10 minutes and then mounted under a coverslip in buffered glycerol (pH 7.0). The slides were given only a code number while being examined under the fluorescence microscope and decoded afterwards. All slides were examined by one person and recorded as positive when several to many definite specifically yellow-green fluorescent cells were seen; doubtful when only 1 or 2 fluorescent cells were present; and negative when no fluorescent cells were found.

Isolation of virus. For the isolation of influenza virus from throat washings, the method of amniotic inoculation as described by Beveridge and Burnet(6) was followed. 1,000 units of penicillin and 500 μg of streptomycin were added to each ml of inoculum.

Hemagglutination - inhibition (HI) tests were performed by the technic described as the Standard Reference Test for Influenza Studies(7). All sera were inactivated at 56°C for 30 minutes, then treated with 4 volumes of crude cholera vibrio filtrate for 18 hours at 37°C to remove the non-specific inhibitors(8). For each test, 4 strains of viral antigens were used: PR8, Fm_1, Lee B, and a current strain of either influenza A or B viruses isolated during the epidemics. A 4-fold or greater increase of antibody titer in the convalescent serum was considered significant.

Analysis of laboratory data. The coded nasal smears stained with fluorescent antibody solutions were usually examined within 24 hours after specimens were collected. The results were then decoded and recorded. Two to 3 weeks later, when acute and convalescent sera were available and HI tests and virus isolation experiments completed, they were compared with the results from the examination of the smears. Influenzal infection was considered confirmed in those patients who had a 4-fold or greater rise of HI antibody titer during convalescence or a positive virus recovery from the nasal washings. Degree of positive correlation as expressed by the ratio of number of positive nasal smears to total number of confirmed cases of influenza is a measure of sensitivity of the test of examination of nasal smears by means of fluorescein-labeled antibody.

Results. It was not the aim of this study to carry out a detailed epidemiological survey of influenza but to attempt to develop a simple, rapid diagnostic method for this disease. Therefore, only a limited number of patients was selected, enabling us to undertake a complete laboratory diagnosis on each patient.

Influenza A-prime epidemics occurred in New England during 1952-3. Twenty patients admitted to the Harvard Stillman Infirmary between the last week of Jan. and the first 2 weeks of Feb. 1953, were studied. The results are summarized in Table I. For fluorescein-labeled antibody staining, only the conjugate against the Farrington strain of influenza virus was used, because this was the only conjugate available at that time. This strain of virus is closely related to the classical PR8 strain of influenza A virus, as reported previously(10). In the hemagglutination-inhibition tests, 4 strains of influenza viral antigens (PR8, Fm_1, 53-Ben and Lee) were used. Significant rise of antibody titer of 4-fold or greater during convalescence was seen only in the A-prime virus such as Fm_1 and the 53-Ben; the latter was isolated during the epidemic. Occasionally a concomitant 4-fold or greater rise of antibody titer against PR8 strain also occurred. However, no case had a significant antibody rise against the Lee strain of influenza B virus. Therefore only data of hemagglutination-inhibition tests on A-prime viruses are tabulated.

TABLE I. Results on Examination of 20 Patients during an Influenza A′ Epidemic.

Clinical diagnosis	Nasal smears*	Virus isolation	HI against A′, –fold rise
Influenza	+	+	32
"	+	—	64
"	—	+	8
Mononucleosis	—	—	0
Influenza	+	—	0
"	—	nd	4
"	+	—	4
"	+	+	8
"	+	—	16
"	+	+	8
"	—	+	32
"	+	—	4
"	—	—	64
"	+	—	128
"	—	—	8
"	+	—	32
Acute tonsillitis	—	—	2
Influenza	+	+	4
"	+	+	4
"	+	+	8

* Stained with fluorescent antibody solution against influenza A virus.

In only 13 cases nasal smears had definite specific fluorescent cells permitting a positive diagnosis of influenza A-prime infection (Table I). The diagnosis in 12 of these positive cases was confirmed by rise of antibody

TABLE II. Results of Rapid Diagnosis of Human Influenza A′ Infection by Fluorescent Antibody Staining of Nasal Smears.

Smears stained with fluorescent antibody	Positive virus recovery or 4-fold rise of HI	2-fold rise or no rise of HI	Total
Positive	12†	1	13
Negative	5	2*	7
Total	17	3	20

Positive smears: 12/17 = 71%

* 1 case of infectious mononucleosis. 1 case of acute tonsillitis.

† 6 cases with positive virus recovery.

TABLE III. Results of Rapid Diagnosis of Human Influenza B Infection by Fluorescent Antibody Staining of Nasal Smears.

Nasal smears from:	Smears stained with fluorescent antibody	Confirmed* influenza	Unconfirmed† influenza	Total
Normal saline nasal washings	Positive	10‡	1	11
	Negative	16	5	21
	Positive smears: 10/26 = 38%			
Nutrient broth nasal washings	Positive	1	0	1
	Negative	5	1	6
	Positive smears: 1/6 = 17%			
Swabbing of nasal turbinate	Positive	0	0	0
	Negative	4§	1	5
	Positive smears: 0			

* Confirmed by a 4-fold or greater rise of HI antibody titer during convalescence and/or virus isolation.

† 2-fold or no rise of HI antibody titer during convalescence.

‡ Including 2 cases of influenza A.

§ Including one case in which smears from swab method were negative but smears from saline nasal washings were positive.

during convalescence, and from 6 of them also an A-prime virus was recovered. Of 7 cases with negative nasal smears, 5 showed a rise in HI antibody, while 2 cases also had positive virus isolation Of the remaining 2 negative nasal smear cases, both were negative for virus isolation and one had a 2-fold rise of HI antibody. The clinical diagnoses of these 2 patients were infectious mononucleosis and acute tonsillitis.

Taking the 4-fold or greater HI antibody rise with or without positive virus isolation as the evidence for influenza infection, out of 17 confirmed cases, 12, or 71% showed positive smears (Table II).

Study of acute febrile illnesses. No influenza was encountered in the winter of 1953-4. However, 23 cases of various types of acute febrile illness or upper respiratory infections including 3 cases of clinical influenza were chosen at random for this study between late Nov. 1953 to Feb. 1954. In these 23 cases no rise of HI antibody against either influenza A or B viruses occurred. However, one patient with acute upper respiratory infection had a positive nasal smear when stained with influenza A′ fluorescent antibody. Therefore, one false positive diagnosis was encountered among 23 non-influenzal cases.

Influenza B epidemic. In this study, a comparison of normal 0.85% saline solution and nutrient broth for nasal washings as well as a study of the use of cotton swabs for nasal smears was undertaken. The best results were obtained from nasal smears using normal saline for nasal washing (Table III); even so, only in 38% of these cases were virus-containing cells found in nasal smears under the fluorescence microscope. The nutrient broth nasal washings produced 1 positive out of 6 specimens. Nasal turbinate swabs from 4 cases were negative. Smears made from nutrient broth nasal washings or swabs had far fewer cells present than those prepared from saline washings. When there were too few cells in the smears, the chance of detecting cells containing influenza antigen was evidently greatly reduced.

Discussion. From the foregoing data, it is evident that a rapid diagnosis of human influenzal infection by means of fluorescein-labeled antibody staining of cells washed out from the nose is feasible. This method is simple and practical; and a diagnosis of influenza could be made on the day the specimens were collected. The shortcoming lies in a lower sensitivity than the hemagglutination-inhibition test. In attempting to explain the lower sensitivity of this rapid diagnostic method, it is possible that we have not been able to examine the patients early enough in their illness for nasal smears. From the experience in the study of experimental influenzal infection in ferrets, the period of positive nasal smears is limited to 2 to 3 days of the comparatively short febrile period of the illness(3). No fluorescent cells were found in the smears before the fever started, probably because onset of fever usually coincides with desquamation of the infected ciliated epithelium into the nasal passage. The reason for the failure to detect cells containing viral antigens in nasal smears after subsidence of fever is not clear, since virus-recovery is still possible. Since patients in our study were healthy young male students, they usually did not seek medical care until they had had a fever and physical discomfort for about 24 to 48 hours, and were generally seen on the third day of their illness. This delay in the time of collection of the nasal washings may account for many of the negative smears. On the other hand, we do not know whether the pathogenesis of influenzal infection in human beings is similar to that in ferrets. It is likely that the desquamation of infected nasal epithelium in human patients may not be as extensive as in ferrets, for in the cases of human influenza uncomplicated by bacterial infections, the epithelium of the bronchioles is intact(9).

The fluorescent antibody solutions used in these studies were not made from current epidemic strains of influenza virus. However, they could be used because of known cross reactions in fluorescent staining among different strains of influenza A virus due to the presence of a common soluble antigen(10,11). Presumably the same is true for influenza B virus. The significant difference between sensitivity of influenza A epidemic study (71% positive) and influenza B epidemic study (38% positive) could be due to a less potent fluorescent conjugate prepared from the Lee strain of influenza B virus or to a milder B infection with less epithelial desquamation.

As yet, there is no perfect diagnostic method for influenzal infections. Even serological tests or isolation of virus do not detect 100% of the infections. The present method is less sensitive than the hemagglutination-inhibition. It is hoped that sensitivity may be increased with more experience. However, if therapeutic agents for viral infections ever become available, retrospective diagnostic tests might become impractical and a rapid diagnosis of the disease becomes desirable.

Summary. A specific rapid diagnosis of human influenzal infection by staining smears of nasal washings with fluorescein-labeled antibody solution is described. This method was applied to 20 patients during an outbreak of influenza A-prime infections, to 44 cases during an outbreak of influenza B, and to 23 cases of various acute febrile or respiratory illnesses. Taking a rise of 4-fold or greater in titer of hemagglutination-inhibition (HI) antibody during convalescence, or the recovery of virus from human nasal washings as diagnostic criteria for influenzal infection, the use of fluorescent antibody had a sensitivity of 71% in an influenza A-prime and 38% in an influenza B epidemic One false positive diagnosis was encountered among 23 non-influenzal infections. The use of labeled antibody on smears of nasal washings is less sensitive than the HI test, but a positive diagnosis could be made on the day the specimens were collected, while the HI test requires a delay of 10 to 14 days for collection of convalescent serum.

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